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PRINCIPAL INVESTIGATOR: Gregory J. Hannon, Ph.D.

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

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Gregory J. Hannon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

E-Mail: hannon@cshl.org

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The broad goal of this project is to develop genome-wide RNAi approaches in mammals and to apply these to the discovery of new therapeutic targets for cancer. Specifically, we will generate a library of short hairpin RNA expression constructs (shRNA) that ultimately correspond to every gene in the human genome. These will be made available as a public resource and used internally to screen for genes that are essential to the survival of breast cancer cells but which are dispensable for the survival of normal cells. A subset of these might prove suitable as therapeutic targets for breast cancer therapy. During the first year of funding, two things have become clear. First, although they were not in place at the time of submitting this application, we have largely developed the technologies necessary to pursue the above goal. Second, funding in the Innovator award falls far short of that necessary to achieve the goal. Relevant to the last point, we have been able to leverage the Innovator award with several other funding sources to create a program, which is capable of meeting the proposed goal.

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INTRODUCTION

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BODY

Progress toward developing the technology necessary for genome-wide RNAi in mammals (these were funded in part by the Innovator award and also by P01 from the NCI)

Stable Suppression of Gene expression by RNAi in mammalian cells

(see attached manuscript as appendix 1)

With our discovery that endogenously encoded small RNAs (microRNAs) enter the RNAi pathway and suppress gene expression at the level of protein synthesis, we considered the possibility that remodeling miRNAs might allow us to manipulate their target specificity. We named these synthetic miRNAs, generically, short hairpin RNAs or shRNAs. We demonstrated that chemically or enzymatically synthesized shRNAs could trigger sequence specific silencing in human and mouse cells (both normal and transformed) and that stable integration of shRNA-expression vectors into the genome permitted continuous suppression of gene expression in cell lines. This work was performed prior to the funding of the Innovator award, but provides important background for subsequent results.

RNAi in living animals

(see attached manuscript, Appendix 2)

We wished to determine whether RNAi, and specifically shRNAs and siRNAs, could induce sequence specific silencing in adult mammals. This was relevant for two reasons. First, the future use of RNAi as a therapeutic tool necessitates that the pathways be active in adult tissues. Second, we hoped to use the library of shRNA constructs that we were designing to do in vivo screens for potential therapeutic targets. To address this question, we formed a collaboration with Mark Kay at Stanford University to test by hydrodynamic transfection whether RNAi functioned in livers of adult mice. This procedure can essentially be considered as a transient co-transfection experiment into the somatic tissues of an adult mammal. Remarkably, shRNA expression constructs could suppress a targeted marker gene by up to 98%. This was the first demonstration that RNAi pathways were operable in the adult and led to experiments to try to model tumor suppressor mutations in vivo in organs reconstituted from stem cells.

RNAi as a genetic tool in vivo – epialleles to study the consequences of hypomorphic mutations in tumor suppressor genes

(see attached manuscript appendix 3)

Tumorigenesis occurs via a stepwise pathway in which the accumulation of multiple genetic lesions provides the essential properties of the cancer cell. Mouse models have been instrumental in recapitulating some of these steps through the creation of genetic mutants. This is a long and technically challenging process, but the outcome has often been extremely valuable. We undertook an effort to create a more rapid methodology that would allow reduction of tumor suppressor pathways in vivo.

Scott Lowe's laboratory had devised a very effective scheme for examining genetic interactions in a model of Burkit's Lymphoma wherein a germline Em-myc oncogene is combined with additional mutations to examine interactions with, for example, the p53 pathway or suppressors of programmed cell death. Although initial studies used transgenics and breeding to create the desired combinations, a more rapid procedure was subsequently implemented, which began with hematapoeitic stem cells from the Em-myc transgenics. These cells were further modified by retroviral infection in vitro and then transferred into irradiated recipients in which the modified stem cells repopulate the immune system. Consequences of genetic interactions can then be read out in this mosaic model.

We teamed with Scott and his laboratory to test whether shRNAs could be used to create loss-of-function alleles in this context. We began by focusing on a tumor suppressor that had been characterized in this system, p53. Several interesting outcomes emerged from this study, which are described in detail in the attached paper. In summary, we demonstrated that shRNAs could be used to stably engineer stem cells in vitro and that phenotypic effects of suppression

were evident in the reconstituted organ system in vivo. Second, we demonstrated that one could use RNAi to create an "epiallelic series" in which different shRNAs suppress the gene of interest to different levels. Not only were different levels of suppression relevant for phenotypic endpoints in vitro but different tumor suppressor hypomorphs created distinct outcomes in vivo. This has profound implications for how we think of the evolution of tumor cells.

A great deal of work in recent years has revealed homeostatic mechanisms that act to counter many oncogene and tumor suppressor mutations. For example myc or ras activation can lead to the elimination of mutant cells through the irreversible endpoints of apoptosis or senescence, respectively. Based upon these observations, one can devise a scheme for progression toward the cancer cell which involves multiple, stepwise alterations, in which small changes in flux through oncogenic pathways is compensated by small changes in the response of tumor suppressor mechanisms. Alternatively, it is possible that partial loss of tumor suppressor function creates a selective advantage which, in effect, get the ball rolling in the transformation process. This is akin to the notion of selection for intermediate forms that can be drawn from evolutionary biology. Consider the basic question : How do we select for stepwise loss of both alleles of a tumor suppressor gene if loss of one allele provides no selective advantage? Our data indicate that partial loss of function for tumor suppressor genes does create a selective advantage, and this is now being borne out for TS genes other than p53.

All of our initial studies have been done for convenience in the Lymphoma model; however, we are now pursuing similar studies using mammary glands reconstituted from stem cells in a collaboration with Senthil Muthuswamy at CSHL.

Germline transmission of RNAi in mammals

(see manuscript appendix 4)

Obviously, the previous two sections led to the possibility that RNAi could be used to created continuous strains of mice in which a gene of interest was stably suppressed by RNAi. In collaboration with Tom Rosenquist, who heads the transgenesis facility at SUNY, Stony Brook, we created transgenic mice carrying an shRNA corresponding to a DNA repair gene, Neil1. Significantly, this shRNA stably suppressed the expression of Neil1 in ES cells and in F1 mice derived from germline transmitting ES cell chimeras. More recently, we have created mice, using lentivirus to deliver the construct sub-zonally, that express the strong p53 shRNA and the creation of mice expressing the weaker p53 epialleles is in progress.

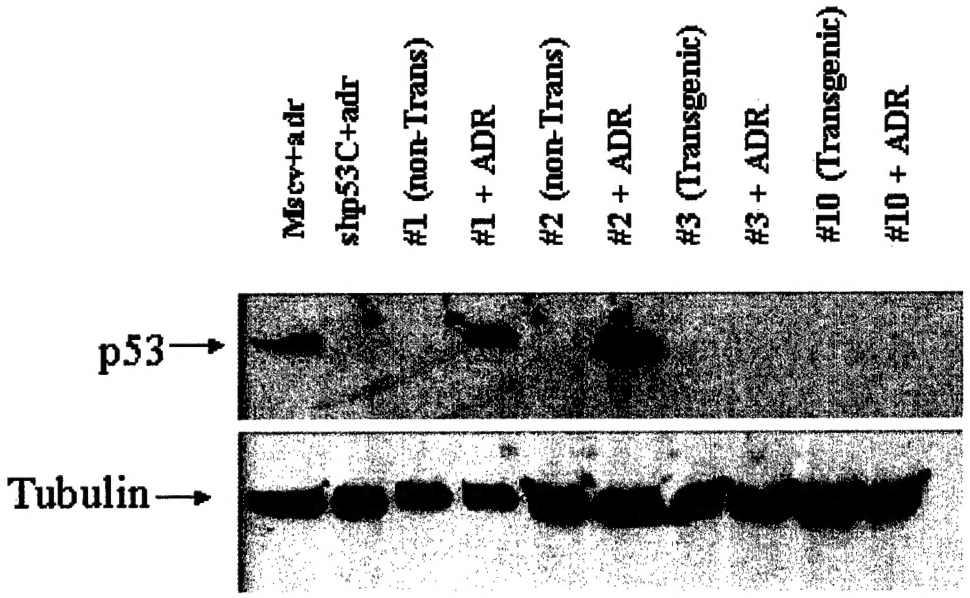


Figure 1. Western analysis of endogenous p53 in dermal fibroblasts of shp53C lentiviral transgenic mice (#'s 3 and 10) and non-transgenic littermate controls (#'s 1 and 2), treated with adriamycin. Lanes 1 and 2 are MEFs infected with either mscv or shp53C and treated with adriamycin.

Studies on the mechanism of RNAi

All of the technology described above was built upon studies of the RNAi mechanism. While these studies are funded by an R01, they benefit from the Innovator award, and it is acknowledged as general support for the P.I. Attached as appendix 5 is a manuscript that describes two additional components of the RNAi machinery, including the Drosophila homolog of the Fragile X mental Retardation Protein. A submitted manuscript (not yet included) demonstrates that the same complex that exists in Drosophila cells also forms with siRNAs in mammalian cells.

A genome-wide RNAi library

Over the last 18 months, there have emerged two major methods for triggering RNAi in mammalian cells. These are transient silencing using siRNAs or stable or transient silencing using shRNAs. Both of these approaches have been validated in numerous publications. In considering how to construct a genome-wide RNA library for human cells, we examined both options. Our choice of the latter reflects several factors. First and foremost, shRNA expression constructs can be propagated and thus provide a limitless supply of material for public distribution. Second, many phenotypes, especially those relevant to breast cancer, require examination of cells over a long time frame. Third, shRNAs offer the flexibility to examine the consequences of silencing both

in vitro and in vivo. Generation of the library is proceeding as a phased project with funding coming in part from the Innovator award and in part from other public and commercial sources (NCI, Merck, Oncogene Sciences). No funding mechanism has been permitted to place any restrictions on library distribution.

Gene selection – Phase I, 10,000 cancer related genes

The overall philosophy of which genes to target was discussed in Washington with the National Cancer Institute. The list comprised several functionally chosen groups, such as kinases, proteolysis genes, phosphatases, GPCRs and trafficked proteins that are considered accessible to medicinal chemistry. In addition, we prioritized genes based upon cellular processes such as DNA repair, cell-cycle and growth control. Finally, we added lists of genes, which had been deemed relevant to cancer in a number of ways. For example, a group of investigators, including Joan Brugge, Ed Harlow and Josh LeBaer at Harvard have hand-collated 1000 genes which are implicated in breast cancer. We have also included genes based upon collated microarray data on breast and lung cancer. Combining all of these sources of information led to a list of ~9,300 high priority genes. We are currently gathering the final ~700 genes to fill out the Phase I library.

shRNA optimization

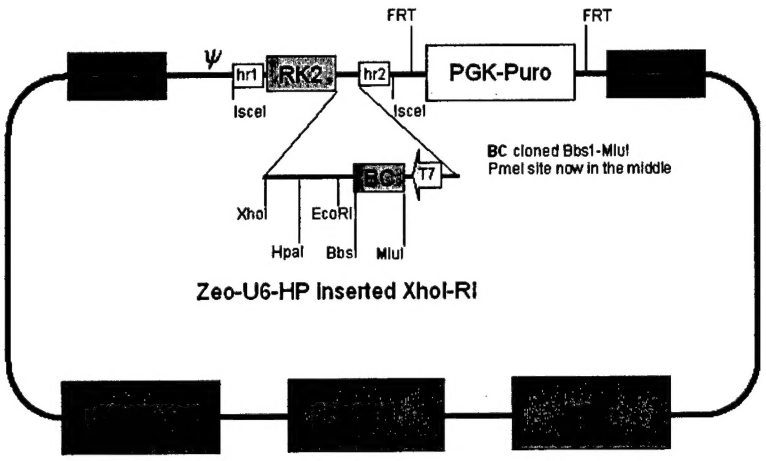
We tested carefully a large number of different strategies to achieve an optimal configuration for shRNA expression and structure. This ultimately translated into a 29 nucleotide hairpin with a simple 4 base loop. This is expressed from the U6 snRNA promoter and is preceded by the U6 leader sequence. We continue to update our strategy and have just begun tiling experiments with 4 genes to attempt to derive rules for choosing optimal shRNAs.

Vector construction and validation

In collaboration with Steve Elledge (Baylor), we constructed a flexible vector system for harboring the shRNA library. We have demonstrated that this vector can transfer shRNA inserts to a recipient plasmid by bacterial mating with ~100% efficiency. We have also validated transfers in multi-well formats suitable for moving subsets of or even the entire library. The original vector design had to be modified to remove loxP sites to avoid intellectual property restrictions placed upon us by Dupont. Persistant, although solvable problems, include the use of Zeocin and the inclusion of FRT sites. We are in the process of negotiating with Salk and Invitrogen to overcome these barriers to distribution. We are also undertaking a pilot project to replace Zeocin with Chloramphenical resistance by recombination. If this becomes necessary, we don't see this creating a significant delay in distribution. At present, Invitrogen has agreed to a 5% royalty

to permit distribution and we are now finalizing that agreement. The second-generation library will not contain zeocin resistance.

Figure 1. Vector system for library construction



shRNA library construction

We have purchased ~40,000 oligonucleotides corresponding to the 9,300 targeted genes. Initial purchases were from Illumina. The performance of the first 10,000 oligos was satisfactory but certainly less than optimal. The performance of the second 10,000 oligos was horrendous. Fully 30% of the 96 well plates that were delivered produced complete failures in our cloning procedure. The performance on the remainder was also abysmal. Illumina could provide no quality control data to assure us that the material that they had shipped were even oligonucleotides. This caused a significant delay and waste of resources, from which we are still recovering. Illumina did provide a refund of ~$100,000, in consideration for this failure. However, this was significantly less than the cost of demonstrating the low quality of their oligos. We have now changed suppliers to Sigma-Genosys and see much better and more consistent performance.

To date, we have sequence verified >22,000 individual shRNAs. Overall library statistics as of 6/09 are shown below. These have been compressed into 96 and 384 well plates and also collected into 384 unit pools. Disribution to several collaborators has begun.

Total Number of Accessions designed	9446
Total Number of Accessions (3 or more)	4489
Total Number of Accessions (2)	2468
Total Number of Accessions (1)	1560
Total Number of Accessions (1 or more)	8517
Total Number of Accessions No Data	929
Total Number of Oligos designed	41278
Total Number of verified human shRNAs	22616
Total Number of mouse designed	11200
Total Number of verified mouse shRNAs	6127

Thus far, ~4500 genes are completed with >3 shRNAs per gene and more than 8,500 genes are covered by at least one construct. More than 6,000 shRNAs show 100% identity to mouse.

Each of the verified shRNAs is associated with a ~60 nucleotide barcode. Sequence analysis suggests that these are >95% unique. We have not yet decided how to handle the 5% which are associated with more than one shRNA, but given the high probability that the library will be most often used in restricted pools, we don't feel an immediate need to replace those shRNAs with redundant barcodes until the first phase of the project is complete. For now, we are printing arrays under a contract with Agilent to permit following the library through population genetic experiments.

Given steady progress, we anticipate finishing this set of genes within the next couple of months.

Working with Rosetta Inpharmatics, we have validated in pilot experiments the use of in situ synthesized oligonucleotides for library construction. Within the next two weeks, CSHL should sign an agreement with Merck that permits access to this technology for shRNA library construction. This agreement does not restrict distribution of clones derived from Rosetta oligos in any way. We are now in the process of testing in situ synthesized oligonucleotides for constructing a very deep set of shRNAs (40 per gene) corresponding to human kinases and for constructing a set of shRNAs (10 per gene) corresponding to ~4,400 mouse genes that were prioritized based upon cancer relevance. These will be constructed in a zeocin-free version of the vector.

Library Distribution

We have signed a public distribution agreement for the library with Open Biosystems and are in negotiations with two other distribution companies.

Clone Validation

We are developing marker-based approaches to validating the shRNA expression cassettes. We and two other groups have shown that the efficacy of shRNAs can be predicted based upon their ability to suppress the activity of a linked marker in a fusion transcript. We are now testing high throughput cloning approaches to creating luciferase fusions for testing shRNAs in co-transfection experiments. These are based upon combining ligation-independent cloning with the ability to create long overhangs using PCR and chimeric DNA-2'O-methyl RNA oligos. We should have a good handle on this process within the next month for ~10 genes. Should this phase be successful, we will test it on the set of human kinases. Ultimately, we may be ably to combine our approach with the MGC to validate the shRNA set more broadly.

Library Screening

This will commence shortly and will hopefully be the subject of next year's progress report.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrate that remodeled miRNAs can induce RNAi in mammalian cells
- Stable silencing in mammalian cells by RNAi
- RNAi works in adult mammals
- RNAi can be used in stem cells
- Modified stem cells can reconstitute an organ system and retain suppression
- Development of new methods for examining the consequences of tumor suppressor hypomorphs
- Germline transmission of RNAi
- Design of numerous RNAi resources including vectors and public web-based informatic tools

- Construction and sequence verification of ~22,000 shRNA expression vectors to cancer related genes

- Design of microarray based procedures for following the library in populations

REPORTABLE OUTCOMES

- Manuscripts (5 attached)

- Vector systems for RNAi (both transient and viral vectors, distributed to ~1000 labs so far)

- Additional funding for library construction from public and private sources

- A genome-wide RNAi library (~2/3 complete)

CONCLUSIONS

RNAi has emerged over the last two years as a powerful tool for experimental manipulation of gene expression and as a potential therapeutic strategy. We have made substantial progress toward validating the use of RNAi in mammals and have contributed key reagents to the scientific community. Notably, we are on the verge of our first genetic screens, which will scan a substantial fraction of the human genome for potential therapeutic targets for cancer.

Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

Patrick J. Paddison,[1] Amy A. Caudy,[1] Emily Bernstein,[2,3] Gregory J. Hannon,[1,2,4] and Douglas S. Conklin[2]

[1]Watson School of Biological Sciences, [2]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA; [3]Graduate Program in Genetics, State University of New York at Stony Brook, Stony Brook, New York 11794, USA

RNA interference (RNAi) was first recognized in *Caenorhabditis elegans* as a biological response to exogenous double-stranded RNA (dsRNA), which induces sequence-specific gene silencing. RNAi represents a conserved regulatory motif, which is present in a wide range of eukaryotic organisms. Recently, we and others have shown that endogenously encoded triggers of gene silencing act through elements of the RNAi machinery to regulate the expression of protein-coding genes. These small temporal RNAs (stRNAs) are transcribed as short hairpin precursors (~70 nt), processed into active, 21-nt RNAs by Dicer, and recognize target mRNAs via base-pairing interactions. Here, we show that short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in cultured *Drosophila* and mammalian cells. shRNAs can be synthesized exogenously or can be transcribed from RNA polymerase III promoters in vivo, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing.

[*Key Words*: RNAi; gene silencing; miRNA; shRNA; siRNA]

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An understanding of the biological role of any gene comes only after observing the phenotypic consequences of altering the function of that gene in a living cell or organism. In many cases, those organisms for which convenient methodologies for genetic manipulation exist blaze the trail toward an understanding of similar genes in less tractable organisms, such as mammals. The advent of RNA interference (RNAi) as an investigational tool has shown the potential to democratize at least one aspect of genetic manipulation, the creation of hypomorphic alleles, in organisms ranging from unicellular parasites (e.g., Shi et al. 2000) to mammals (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000).

Although *Caenorhabditis elegans* has, for some time, been well developed as a forward genetic system, the lack of methodologies for gene replacement by homologous recombination presented a barrier to assessing rapidly the consequences of loss of function in known genes. In an effort to overcome this limitation, Mello and Fire (Fire et al. 1998), building on earlier studies (Guo and Kemphues 1995), probed the utility of antisense RNA as a method for suppressing gene expression in worms. Through these efforts, they found that double-stranded RNA (dsRNA) was much more effective than antisense RNA as an inducer of gene silencing. Subsequent studies have shown that RNAi is a conserved biological response that is present in many, if not most, eukaryotic organisms (for review, see Bernstein et al. 2001b; Hammond et al. 2001b).

As a result of biochemical and genetic approaches in several experimental systems, the mechanisms underlying RNAi have begun to unfold (for review, see Bernstein et al. 2001b; Hammond et al. 2001b). These suggest the existence of a conserved machinery for dsRNA-induced gene silencing, which proceeds via a two-step mechanism. In the first step, the dsRNA silencing trigger is recognized by an RNase III family nuclease called Dicer, which cleaves the dsRNA into ~21–23-nt siRNAs (small interfering RNAs). These siRNAs are incorporated into a multicomponent nuclease complex, RISC, which identifies substrates through their homology to siRNAs and targets these cognate mRNAs for destruction.

Although it was clear from the outset that RNAi would prove a powerful tool for manipulating gene expression in invertebrates, there were several potential impediments to the use of this approach in mammalian cells. Most mammalian cells harbor a potent antiviral response that is triggered by the presence of dsRNA viral

[4]Corresponding author.
E-MAIL hannon@cshl.org; FAX (516) 367-8874.
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replication intermediates. A key component of this response is a dsRNA-activated protein kinase, PKR, which phosphorylates EIF-2α, inducing, in turn, a generalized inhibition of translation (for review, see Williams 1997; Gil and Esteban 2000). In addition, dsRNA activates the 2′5′ oligoadenylate polymerase/RNase L system and represses IκB. The ultimate outcome of this set of responses is cell death via apoptosis.

Therefore, it came as a welcome surprise that dsRNA could induce sequence-specific silencing in mammalian embryos, which apparently lack generalized responses to dsRNA (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). Indeed, microinjection of dsRNA into mouse zygotes could specifically silence both exogenous reporters and endogenous genes to create anticipated phenotypes. Subsequently, these observations were extended to embryonic cell lines, such as embryonic stem cells and embryonal carcinoma cells, which do not show generic translational repression in response to dsRNA (Billy et al. 2001; Yang et al. 2001; Paddison et al. 2002). However, restriction of conventional RNAi to these few embryonic and cell culture systems would place a significant limitation on the utility of this approach in mammals.

Tuschl and colleagues first showed that short RNA duplexes, designed to mimic the products of the Dicer enzyme, could trigger RNA interference in vitro in *Drosophila* embryo extracts (Tuschl et al. 1999; Elbashir et al. 2001b,c). This observation was extended to mammalian somatic cells by Tuschl and coworkers (Elbashir et al. 2001a) and by Fire and colleagues (Caplen et al. 2001), who showed that chemically synthesized siRNAs could induce gene silencing in a wide range of human and mouse cell lines. The use of synthetic siRNAs to transiently suppress the expression of target genes is quickly becoming a method of choice for probing gene function in mammalian cells.

Dicer, the enzyme that normally produces siRNAs in vivo, has been linked to RNA interference both through biochemistry and through genetics (Bernstein et al. 2001a; Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001). Indeed, *C. elegans* animals that lack Dicer are RNAi-deficient, at least in some tissues. However, these animals also have additional phenotypic abnormalities. Specifically, they are sterile and show a number of developmental abnormalities that typify alterations in developmental timing. Indeed, the phenotypes of the *Dicer* mutant animals were similar to those previously observed for animals carrying mutations in the *let-7* gene (Reinhart et al. 2000).

The *let-7* gene encodes a small, highly conserved RNA species that regulates the expression of endogenous protein-coding genes during worm development. The active RNA species is transcribed initially as an ~70-nt precursor, which is posttranscriptionally processed into a mature ~21-nt form (Reinhart et al. 2000). Both in vitro and in vivo data from *C. elegans* (Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001) and human cells (Hutvagner et al. 2001) have pointed to Dicer as the enzyme responsible for *let-7* maturation and for the maturation of a similar small RNA, *lin-4* (Grishok et al. 2001). Thus, at least some components of the RNAi machinery respond to endogenously encoded triggers to regulate the expression of target genes.

Recent studies have placed *let-7* and *lin-4* as the founding members of a potentially very large group of small RNAs known generically as micro-RNAs (miRNAs). Nearly 100 potential miRNAs have now been identified in *Drosophila*, *C. elegans*, and mammals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Although the functions of these diverse RNAs remain mysterious, it seems likely that they, like *let-7* and *lin-4*, are transcribed as hairpin RNA precursors, which are processed to their mature forms by Dicer (Lee and Ambros 2001; E. Bernstein, unpubl.).

Since the realization that small, endogenously encoded hairpin RNAs could regulate gene expression via elements of the RNAi machinery, we have sought to exploit this biological mechanism for the regulation of desired target genes. Here we show that short hairpin RNAs (shRNAs) can induce sequence-specific gene silencing in mammalian cells. As is normally done with siRNAs, silencing can be provoked by transfecting exogenously synthesized hairpins into cells. However, silencing can also be triggered by endogenous expression of shRNAs. This observation opens the door to the production of continuous cells lines in which RNAi is used to stably suppress gene expression in mammalian cells. Furthermore, similar approaches should prove efficacious in the creation of transgenic animals and potentially in therapeutic strategies in which long-term suppression of gene function is essential to produce a desired effect.

Results

Short hairpin RNAs trigger gene silencing in Drosophila *cells*

Several groups (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have shown that endogenous triggers of gene silencing, specifically small temporal RNAs (stRNAs) *let-7* and *lin-4*, function at least in part through RNAi pathways. Specifically, these small RNAs are encoded by hairpin precursors that are processed by Dicer into mature, ~21-nt forms. Moreover, genetic studies in *C. elegans* have shown a requirement for Argonaute-family proteins in stRNA function. Specifically, alg-1 and alg-2, members of the EIF2c subfamily, are implicated both in stRNA processing and in their downstream effector functions (Grishok et al. 2001). We have recently shown that a component of RISC, the effector nuclease of RNAi, is a member of the Argonaute family, prompting a model in which stRNAs may function through RISC-like complexes, which regulate mRNA translation rather than mRNA stability (Hammond et al. 2001a).

We wished to test the possibility that we might retarget these small, endogenously encoded hairpin RNAs to regulate genes of choice with the ultimate goal of sub-

verting this regulatory system for manipulating gene expression stably in mammalian cell lines and in transgenic animals. Whether triggered by long dsRNAs or by siRNAs, RNAi is generally more potent in the suppression of gene expression in *Drosophila* S2 cells than in mammalian cells. We therefore chose this model system in which to test the efficacy of short hairpin RNAs (shRNAs) as inducers of gene silencing.

Neither stRNAs nor the broader group of miRNAs that has recently been discovered form perfect hairpin structures. Indeed, each of these RNAs is predicted to contain several bulged nucleotides within their rather short (~30-nt) stem structures. Because the position and character of these bulged nucleotides have been conserved throughout evolution and among at least a subset of miRNAs, we sought to design retargeted miRNA mimics to conserve these predicted structural features. Only the *let-7* and *lin-4* miRNAs have known mRNA targets (Wightman et al. 1993; Slack et al. 2000). In both cases, pairing to binding sites within the regulated transcripts is imperfect, and in the case of *lin-4*, the presence of a bulged nucleotide is critical to suppression (Ha et al. 1996). We therefore also designed shRNAs that paired imperfectly with their target substrates. A subset of these shRNAs is depicted in Figure 1A.

To permit rapid testing of large numbers of shRNA variants and quantitative comparison of the efficacy of suppression, we chose to use a dual-luciferase reporter system, as previously described for assays of RNAi in both *Drosophila* extracts (Tuschl et al. 1999) and mammalian cells (Caplen et al. 2001; Elbashir et al. 2001a). Cotransfection of firefly and *Renilla* luciferase reporter plasmids with either long dsRNAs or with siRNAs homologous to the firefly luciferase gene yielded an ~95% suppression of firefly luciferase without effect on *Renilla* luciferase (Fig. 1B; data not shown). Firefly luciferase could also be specifically silenced by cotransfection with homologous shRNAs. Surprisingly, those shRNAs modeled most closely on the *let-7* paradigm were the least effective inducers of silencing (data not shown). The inclusion of bulged nucleotides within the shRNA stem caused only a modest reduction in potency; however, the presence of mismatches with respect to the target mRNA essentially abolished silencing potential. The most potent inhibitors were those composed of simple hairpin structures with complete homology to the sub-

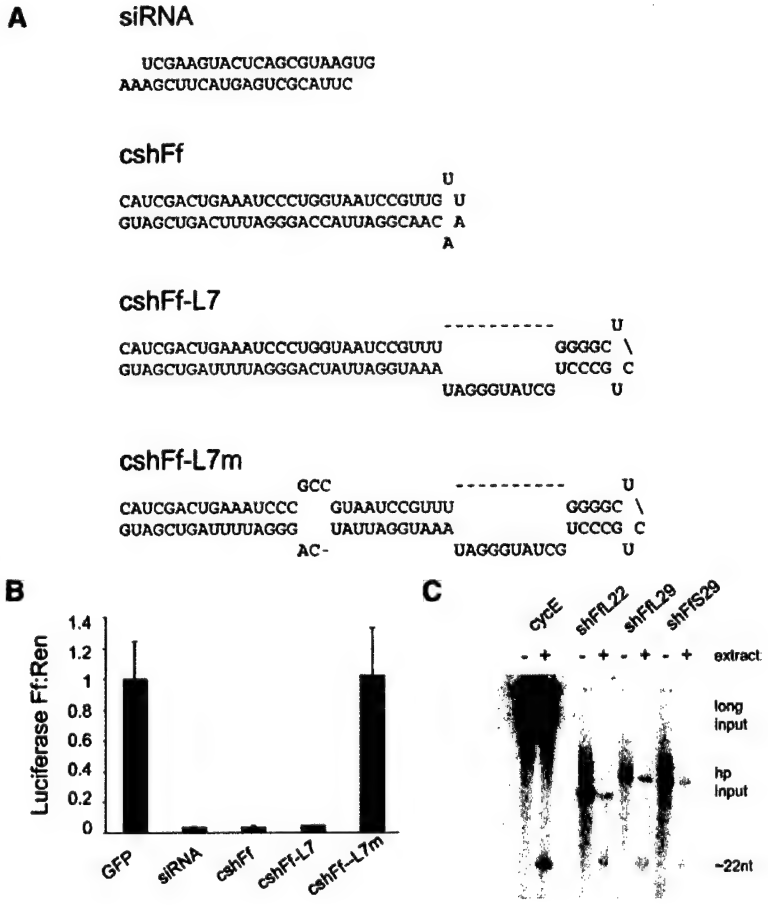

Figure 1. Short hairpins suppress gene expression in *Drosophila* S2 cells. (*A*) Sequences and predicted secondary structure of representative chemically synthesized RNAs. Sequences correspond to positions 112–134 (siRNA) and 463–491 (shRNAs) of Firefly luciferase carried on pGL3-Control. An siRNA targeted to position 463–485 of the luciferase sequence was virtually identical to the 112–134 siRNA in suppressing expression, but is not shown. (*B*) Exogenously supplied short hairpins suppress expression of the targeted Firefly luciferase gene in vivo. Six-well plates of S2 cells were transfected with 250 ng/well of plasmids that direct the expression of firefly and *Renilla* luciferase and 500 ng/well of the indicated RNA. Luciferase activities were assayed 48 h after transfection. Ratios of firefly to *Renilla* luciferase activity were normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation. (*C*) Short hairpins are processed by the *Drosophila* Dicer enzyme. T7 transcribed hairpins shFfL22, shFfL29, and shFfS29 were incubated with (+) and without (-) 0–2-h *Drosophila* embryo extracts. Those incubated with extract produced ~22-nt siRNAs, consistent with the ability of these hairpins to induce RNA interference. A long dsRNA input (cyclin E 500-mer) was used as a control. Cleavage reactions were performed as described in Bernstein et al. (2001a).

strate. Introduction of G-U basepairs either within the stem or within the substrate recognition sequence had little or no effect (Fig. 1A,B; data not shown). Similarly, varying either the loop size from ~4 to 23 bases or the loop sequence (e.g., to mimic *let-7*) also proved neutral (data not shown).

These results show that short hairpin RNAs can induce gene silencing in *Drosophila* S2 cells with potency similar to that of siRNAs (Fig. 1B). However, in our initial observation of RNA interference in *Drosophila* S2 cells, we noted a profound dependence of the efficiency of silencing on the length of the dsRNA trigger (Hammond et al. 2000). Indeed, dsRNAs of fewer than ~200 nt triggered silencing very inefficiently. Silencing is initiated by an RNase III family nuclease, Dicer, that processes long dsRNAs into ~22-nt siRNAs. In accord with their varying potency as initiators of silencing, long dsRNAs are processed much more readily than short RNAs by the Dicer enzyme (Bernstein et al. 2001a). We therefore tested whether shRNAs were substrates for the Dicer enzyme.

We had noted previously that *let-7* (Ketting et al. 2001) and other miRNAs (E. Bernstein, unpubl.) are processed by Dicer with an unexpectedly high efficiency as compared with short, nonhairpin dsRNAs. Similarly, Dicer efficiently processed shRNAs that targeted firefly luciferase, irrespective of whether they were designed to mimic a natural Dicer substrate (*let-7*) or whether they were simple hairpin structures (Fig. 1C). These data suggest that recombinant shRNAs can be processed by Dicer into siRNAs and are consistent with the idea that these short hairpins trigger gene silencing via an RNAi pathway.

Short hairpin activated gene silencing in mammalian cells

RNAi is developing into an increasingly powerful methodology for manipulating gene expression in diverse experimental systems. However, mammalian cells contain several endogenous systems that were predicted to hamper the application of RNAi. Chief among these is a dsRNA-activated protein kinase, PKR, which effects a general suppression of translation via phosphorylation of EIF-2α (Williams 1997; Gil and Esteban 2000). Activation of these, and other dsRNA-responsive pathways, generally requires duplexes exceeding 30 bp in length, possibly to permit dimerization of the enzyme on its allosteric activator (e.g., Clarke and Mathews 1995).

Small RNAs that mimic Dicer products, siRNAs, presumably escape this limit and trigger specific silencing, in part because of their size. However, short duplex RNAs that lack signature features of siRNAs can efficiently induce silencing in *Drosophila* S2 cells but not in mammalian cells (A.A. Caudy, unpubl.). Endogenously encoded miRNAs may also escape PKR surveillance because of their size but perhaps also because of the discontinuity of their duplex structure. Given that shRNAs of <30 bp were effective inducers of RNAi in *Drosophila* S2 cells, we tested whether these RNAs could also induce sequence-specific silencing in mammalian cells.

Human embryonic kidney (HEK293T) cells were cotransfected with chemically synthesized shRNAs and with a mixture of firefly and *Renilla* luciferase reporter plasmids. As had been observed in S2 cells, shRNAs were effective inducers of gene silencing. Once again, hairpins designed to mimic *let-7* were consistently less effective than were simple hairpin RNAs, and the introduction of mismatches between the antisense strand of the shRNA and the mRNA target abolished silencing (Fig. 2A; data not shown). Overall, shRNAs were somewhat less potent silencing triggers than were siRNAs. Whereas siRNAs homologous to firefly luciferase routinely yielded ~90%–95% suppression of gene expression, suppression levels achieved with shRNAs ranged from 80%–90% on average. As we also observe with siRNAs, the most important determinant of the potency of the silencing trigger is its sequence. We find that roughly 50% of both siRNAs and shRNAs are competent for suppressing gene expression. However, neither analysis of the predicted structures of the target mRNA nor analysis of alternative structures in siRNA duplexes or shRNA hairpins has proved of predictive value for choosing effective inhibitors of gene expression.

We have adopted as a standard, shRNA duplexes containing 29 bp. However, the size of the helix can be reduced to ~25 nt without significant loss of potency. Duplexes as short as 22 bp can still provoke detectable silencing, but do so less efficiently than do longer duplexes. In no case do we observe a reduction in the internal control reporter (*Renilla* luciferase) that would be consistent with an induction of nonspecific dsRNA responses.

The ability of shRNAs to induce gene silencing was not confined to 293T cells. Similar results were also obtained in a variety of other mammalian cell lines, including human cancer cells (HeLa), transformed monkey epithelial cells (COS-1), murine fibroblasts (NIH 3T3), and diploid human fibroblasts (IMR90; Fig. 2; data not shown).

Synthesis of effective inhibitors of gene expression using T7 RNA polymerase

The use of siRNAs to provoke gene silencing is developing into a standard methodology for investigating gene function in mammalian cells. To date, siRNAs have been produced exclusively by chemical synthesis (e.g., Caplen et al. 2001; Elbashir et al. 2001a). However, the costs associated with this approach are significant, limiting its potential utility as a tool for investigating in parallel the functions of large numbers of genes. Short hairpin RNAs are presumably processed into active siRNAs in vivo by Dicer (see Fig. 1C). Thus, these may be more tolerant of terminal structures, both with respect to nucleotide overhangs and with respect to phosphate termini. We therefore tested whether shRNAs could be prepared by in vitro transcription with T7 RNA polymerase.

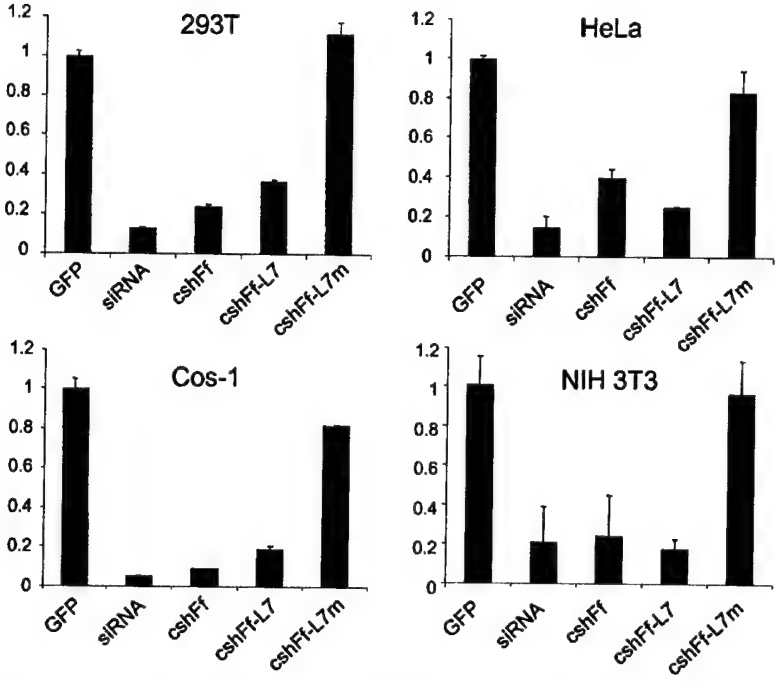

Figure 2. Short hairpins function in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with plasmids and RNAs as in Figure 1 and subjected to dual luciferase assays 48 h posttransfection. The ratios of firefly to *Renilla* luciferase activity are normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation.

Transcription templates that were predicted to generate siRNAs and shRNAs similar to those prepared by chemical RNA synthesis were prepared by DNA synthesis (Fig. 3A,C). These were tested for efficacy both in S2 cells (data not shown) and in human 293 cells (Fig. 3B,D). Overall, the performance of the T7-synthesized hairpin or siRNAs closely matched the performance of either produced by chemical synthesis, both with respect to the magnitude of inhibition and with respect to the relative efficiency of differing sequences. Because T7 polymerase prefers to initiate at twin guanosine residues, however, it was critical to consider initiation context when designing in vitro transcribed siRNAs (Fig. 3B). In contrast, shRNAs, which are processed by Dicer (see Fig. 1C), tolerate the addition of these bases at the 5′ end of the transcript.

Studies in *Drosophila* embryo extracts indicate that siRNAs possess 5′ phosphorylated termini, consistent with their production by an RNase III family nuclease (Bernstein et al. 2001a; Elbashir et al. 2001b). In vitro, this terminus is critical to the induction of RNAi by synthetic RNA oligonucleotides (Elbashir et al. 2001c; Nykanen et al. 2001). Chemically synthesized siRNAs are nonphosphorylated, and enzymatic addition of a 5′ phosphate group in vitro prior to transfection does not increase the potency of the silencing effect (A.A. Caudy, unpubl.). This suggests either that the requirement for phosphorylated termini is less stringent in mammalian cells or that a kinase efficiently phosphorylates siRNAs in vivo. RNAs synthesized with T7 RNA polymerase, however, possess 5′ triphosphate termini. We therefore explored the possibility of synthesizing siRNAs with T7 polymerase followed by treatment in vitro with pyrophosphatase to modify the termini to resemble those of siRNAs. Surprisingly, monophosphorylated siRNAs (data not shown) were as potent in inducing gene silencing as transcription products bearing triphosphate termini (Fig. 3B). This may suggest either that the requirement for monophosphorylated termini is less stringent in mammalian cells or that siRNAs are modified in vivo to achieve an appropriate terminal structure.

Considered together, our data suggest that both shRNAs and siRNA duplexes can be prepared by synthesis with T7 RNA polymerase in vitro. This significantly reduces the cost of RNAi in mammalian cells and paves the way for application of RNAi on a whole-genome scale.

Transcription of shRNAs in vivo by RNA polymerase III

Although siRNAs are an undeniably effective tool for probing gene function in mammalian cells, their suppressive effects are by definition of limited duration. Delivery of siRNAs can be accomplished by any of a num-

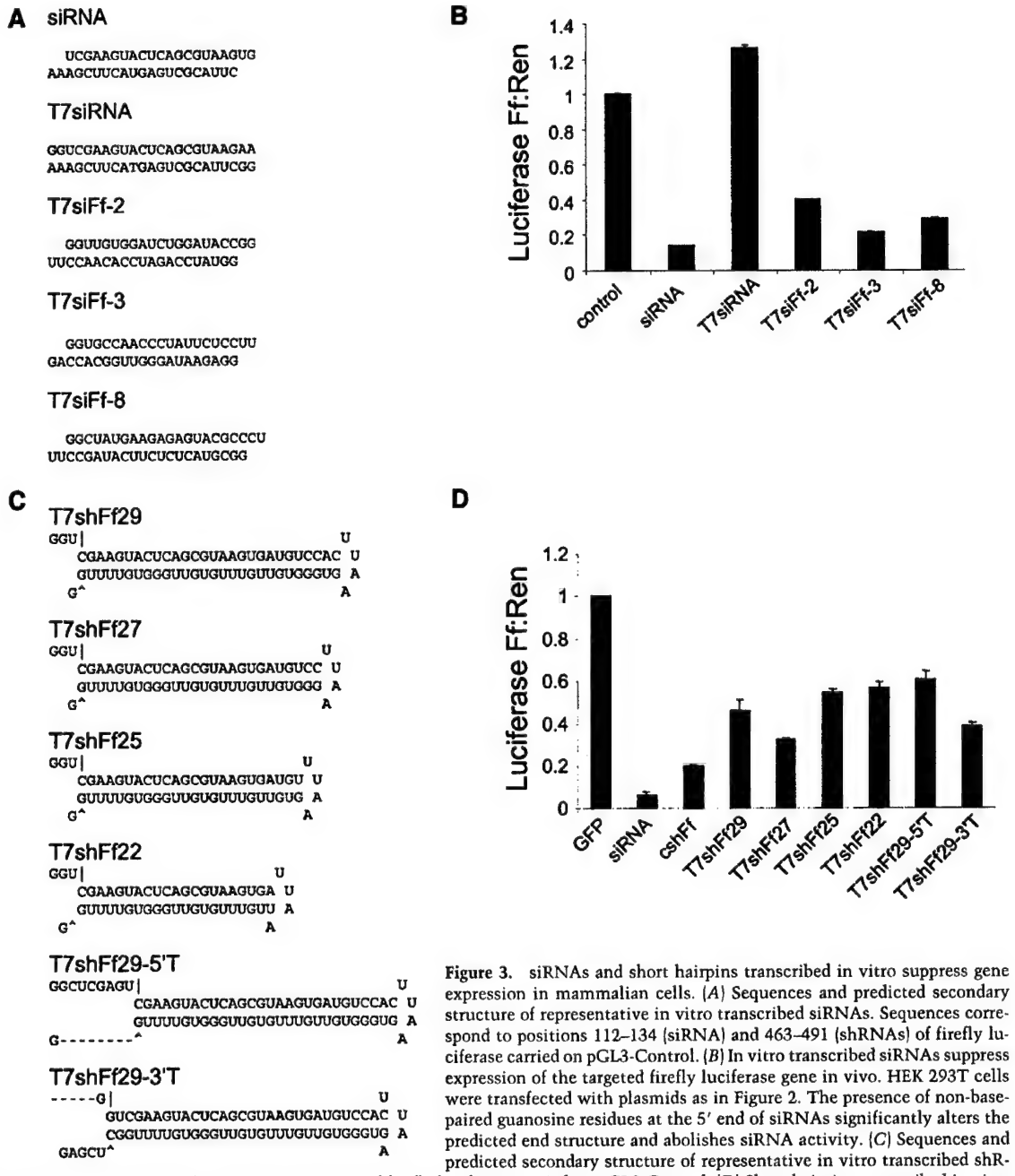

Figure 3. siRNAs and short hairpins transcribed in vitro suppress gene expression in mammalian cells. (*A*) Sequences and predicted secondary structure of representative in vitro transcribed siRNAs. Sequences correspond to positions 112–134 (siRNA) and 463–491 (shRNAs) of firefly luciferase carried on pGL3-Control. (*B*) In vitro transcribed siRNAs suppress expression of the targeted firefly luciferase gene in vivo. HEK 293T cells were transfected with plasmids as in Figure 2. The presence of non-base-paired guanosine residues at the 5′ end of siRNAs significantly alters the predicted end structure and abolishes siRNA activity. (*C*) Sequences and predicted secondary structure of representative in vitro transcribed shRNAs. Sequences correspond to positions 112–141 of firefly luciferase carried on pGL3-Control. (*D*) Short hairpins transcribed in vitro suppress expression of the targeted firefly luciferase gene in vivo. HEK 293T cells were transfected with plasmids as in Figure 2.

ber of transient transfection methodologies, and both the timing of peak suppression and the recovery of protein levels as silencing decays can vary with both the cell type and the target gene (Y. Seger and E. Bernstein, unpubl.). Therefore, one limitation on siRNAs is the development of continuous cell lines in which the expression of a desired target is stably silenced.

Hairpin RNAs, consisting of long duplex structures, have been proved as effective triggers of stable gene silencing in plants, in *C. elegans*, and in *Drosophila* (Ken-

nerdell and Carthew 2000; Smith et al. 2000; Tavernarakis et al. 2000). We have recently shown stable suppression of gene expression in cultured mammalian cells by continuous expression of a long hairpin RNA (Paddison et al. 2002). However, the scope of this approach was limited by the necessity of expressing such hairpins only in cells that lack a detectable PKR response. In principle, shRNAs could bypass such limitations and provide a tool for evoking stable suppression by RNA in mammalian somatic cells.

To test this possibility, we initially cloned sequences encoding a firefly luciferase shRNA into a CMV-based expression plasmid. This was predicted to generate a capped, polyadenylated RNA polymerase II transcript in which the hairpin was extended on both the 5′ and 3′ ends by vector sequences and poly(A). This construct was completely inert in silencing assays in 293T cells (data not shown).

During our studies on chemically and T7-synthesized shRNAs, we noted that the presence of significant single-stranded extensions (either 5′ or 3′ of the duplex) reduced the efficacy of shRNAs (data not shown). We therefore explored the use of alternative promoter strategies in an effort to produce more defined hairpin RNAs. In particular, RNA polymerase III promoters have well-defined initiation and termination sites and naturally produce a variety of small, stable RNA species. Although many Pol III promoters contain essential elements within the transcribed region, limiting their utility for our purposes; class III promoters use exclusively non-transcribed promoter sequences. Of these, the U6 snRNA promoter and the H1 RNA promoter have been well studied (Lobo et al. 1990; Hannon et al. 1991; Chong et al. 2001).

By placing a convenient cloning site immediately behind the U6 snRNA promoter, we have constructed pShh-1, an expression vector in which short hairpins are harnessed for gene silencing. Into this vector either of two shRNA sequences derived from firefly luciferase were cloned from synthetic oligonucleotides. These were cotransfected with firefly and *Renilla* luciferase expression plasmids into 293T cells. One of the two encoded shRNAs provoked effective silencing of firefly luciferase without altering the expression of the internal control (Fig. 4C). The second encoded shRNA also produced detectable, albeit weak, repression. In both cases, silencing was dependent on insertion of the shRNA in the correct orientation with respect to the promoter (Fig. 4C; data not shown). Although the shRNA itself is bilaterally symmetric, insertion in the incorrect orientation would affect Pol III termination and is predicted to produce a hairpin with both 5′ and 3′ single-stranded extensions. Similar results were also obtained in a number of other mammalian cell lines including HeLa, COS-1, NIH 3T3, and IMR90 (Fig. 4; data not shown). pShh1-Ff1 was, however, incapable of effecting suppression of the luciferase reporter in *Drosophila* cells, in which the human U6 promoter is inactive (data not shown).

As a definitive test of whether the plasmid-encoded shRNAs brought about gene silencing via the mammalian RNAi pathway, we assessed the dependence of suppression on an essential component of the RNAi pathway. We transfected pShh1-Ff1 along with an siRNA homologous to human *Dicer*. Figure 5 shows that treatment of cells with *Dicer* siRNAs is able to completely depress the silencing induced by pShh1-Ff1. Addition of an unrelated siRNA had no effect on the magnitude of suppression by pShh1-Ff1 (data not shown). Importantly, *Dicer* siRNAs had no effect on siRNA-induced silencing of firefly luciferase (data not shown). These results are consistent with shRNAs operating via an RNAi pathway similar to those provoked by stRNAs and long dsRNAs. Furthermore, it suggests that siRNA-mediated silencing is less sensitive to depletion of the Dicer enzyme.

The ultimate utility of encoded short hairpins will be in the creation of stable mutants that permit the study of the resulting phenotypes. We therefore tested whether we could create a cellular phenotype through stable suppression. Expression of activated alleles of the *ras* oncogene in primary mouse embryo fibroblasts (MEFs) induces a stable growth arrest that resembles, as a terminal phenotype, replicative senescence (Serrano et al. 1997). Cells cease dividing and assume a typical large, flattened morphology. Senescence can be countered by mutations that inactivate the p53 tumor suppressor pathway (Serrano et al. 1997). As a test of the ability of vector-encoded shRNAs to stably suppress an endogenous cellular gene, we generated a hairpin that was targeted to the mouse *p53* gene. As shown in Figure 6, MEFs transfected with pBabe-RasV12 fail to proliferate and show a senescent morphology when cotransfected with an empty control vector. As noted previously (Serrano et al. 1997), the terminally arrested state is achieved in 100% of drug-selected cells in culture by 8 d posttransfection. However, upon cotransfection of an activated *ras* expression construct with the pShh-p53, cells emerged from drug selection that not only fail to adopt a senescent morphology but also maintain the ability to proliferate for a minimum of several weeks in culture (Fig. 6). These data strongly suggest that shRNA expression constructs can be used for the creation of continuous mammalian cell lines in which selected target genes are stably suppressed.

Discussion

The demonstration that short dsRNA duplexes can induce sequence-specific silencing in mammalian cells has begun to foment a revolution in the manner in which gene function is examined in cultured mammalian cells. These siRNAs (Elbashir et al. 2001a) mimic the products generated by Dicer (Bernstein et al. 2001a) in the initiation step of RNAi and presumably enter the silencing pathway without triggering nonspecific translational suppression via PKR. siRNAs can be used to examine the consequences of reducing the function of virtually any protein-coding gene and have proved effective in provoking relevant phenotypes in numerous somatic cell types from both humans and mice. However, a significant dis-

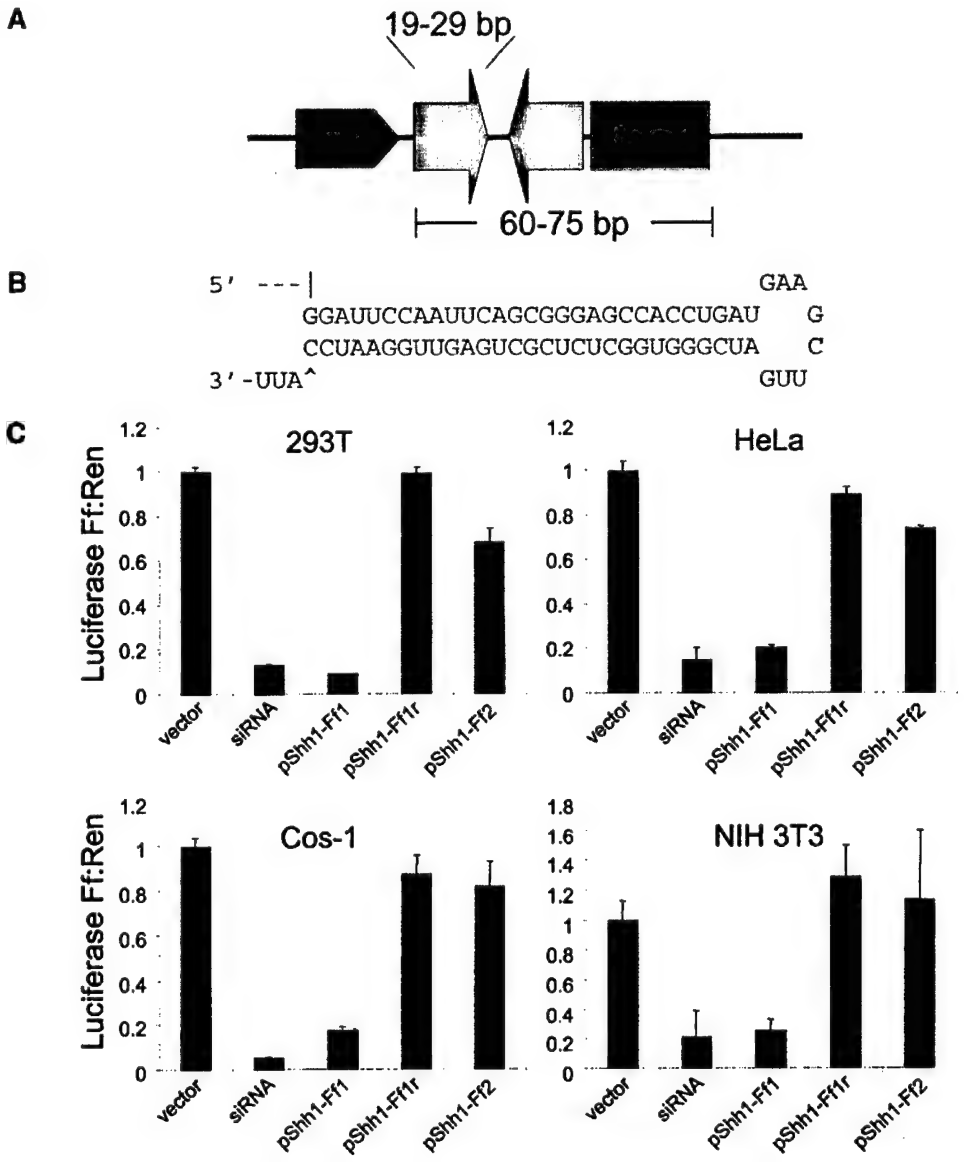

Figure 4. Transcription of functional shRNAs in vivo. (*A*) Schematic of the pShh1 vector. Sequences encoding shRNAs with between 19 and 29 bases of homology to the targeted gene are synthesized as 60–75-bp double-stranded DNA oligonucleotides and ligated into an *Eco*RV site immediately downstream of the U6 promoter. (*B*) Sequence and predicted secondary structure of the Ff1 hairpin. (*C*) An shRNA expressed from the pShh1 vector suppresses luciferase expression in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with reporter plasmids as in Figure 1, and pShh1 vector, firefly siRNA, or pShh1 firefly shRNA constructs as indicated. The ratios of firefly to *Renilla* luciferase activity were determined 48 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

advantage of siRNAs is that their effects are transient, with phenotypes generated by transfection with such RNAs persisting for ~1 wk. In *C. elegans*, RNAi has proved to be such a powerful tool, in part, because silencing is both systemic and heritable, permitting the consequences of altering gene expression to be examined throughout the development and life of an animal. We have therefore sought to expand the utility of RNAi in mammalian systems by devising methods to induce stable and heritable gene silencing. Previously, we have shown that expression of long (~500-nt) dsRNAs could produce stable silencing in embryonic mammalian cells

Figure 5. Dicer is required for shRNA-mediated gene silencing. HEK 293T cells were transfected with luciferase reporter plasmids as well as pShh1-Ff1 and an siRNA targeting human Dicer either alone or in combination, as indicated. The Dicer siRNA sequence (TCA ACC AGC CAC TGC TGG A) corresponds to coordinates 3137–3155 of the human *Dicer* sequence. The ratios of firefly to *Renilla* luciferase activity were determined 26 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

(Paddison et al. 2002); however, the utility of this approach was limited by its restriction to cells that lack endogenous, nonspecific responses to dsRNA, such as PKR.

Recently, a number of laboratories (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have shown that there exist endogenously encoded triggers of RNAi-related pathways, which are transcribed as short hairpin RNAs (stRNAs, or generically miRNAs). Here, we have shown that short hairpin RNAs, modeled conceptually on miRNAs, are potent experimental tools for inducing gene silencing in mammalian somatic cells. These shRNAs can be provided exogenously or can be synthesized in vivo from RNA polymerase III promoters. Not only does this enable the creation of continuous cell lines in which suppression of a target gene is stably maintained by RNAi, but similar strategies may also be useful for the construction of transgenic animals. Thus, short-hairpin-activated gene silencing (SHAGging) provides a complement to the use of siRNAs in the study of gene function in mammalian cells. Finally, the ability to encode a constitutive silencing signal may permit the marriage of shRNA-induced silencing with in vivo and ex vivo gene delivery methods for therapeutic approaches based on stable RNAi in humans.

Materials and methods

Cell culture

HEK 293T, HeLa, COS-1, MEF, and IMR90 cells were cultured in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO BRL). NIH 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated calf serum and 1% antibiotic/antimycotic solution.

RNA preparation

Both shRNAs and siRNAs were produced in vitro using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates

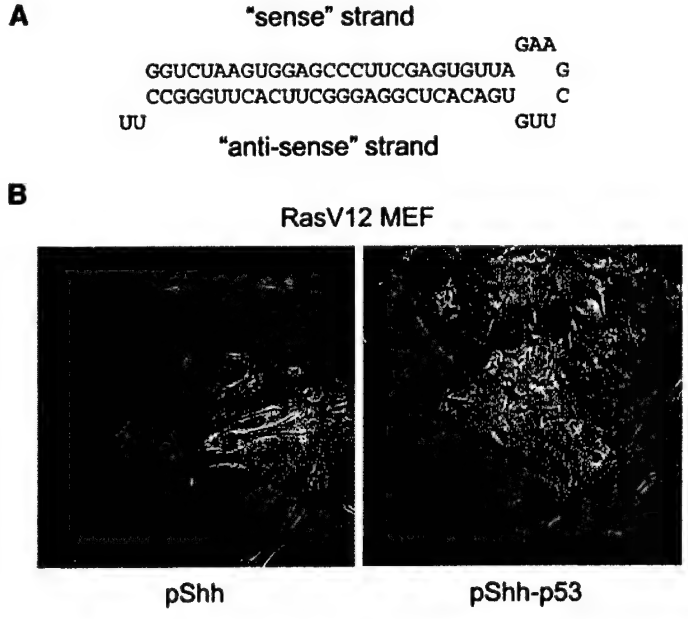

Figure 6. Stable shRNA-mediated gene silencing of an endogenous gene. (*A*) Sequence and predicted secondary structure of the *p53* hairpin. The 5′ shRNA stem contains a 27-nt sequence derived from mouse *p53* (nucleotides 166–192), whereas the 3′ stem harbors the complimentary antisense sequence. (*B*) Senescence bypass in primary mouse embryo fibroblasts (MEFs) expressing an shRNA targeted at *p53*. Wild-type MEFs, passage 5, were transfected with pBabe-RasV12 with control plasmid or with p53hp (5 μg each with FuGENE; Roche). Two days after transfection, cells were trypsinized, counted, and plated at a density of 1×10^5/10-cm plate in media containing 2.0 μg/mL of puromycin. Control cells cease proliferation and show a senescent morphology (*left* panel). Cells expressing the *p53* hairpin continue to grow (*right* panel). Photos were taken 14 d posttransfection.

were designed such that they contained *T7* promoter sequences at the 5′ end. shRNA transcripts subjected to in vitro Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained from Dharmacon, Inc.

Transfection and gene silencing assays

Cells were transfected with indicated amounts of siRNA, shRNA, and plasmid DNA using standard calcium phosphate procedures at 50%–70% confluence in 6-well plates. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids containing firefly luciferase under the control of the SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). Plasmids were cotransfected using a 1:1 ratio of pGL3-Control (250 ng/well) to pRL-SV40. RNAi in S2 cells was performed as previously described (Hammond et al. 2000). For stable silencing, primary MEFs (a gift from S. Lowe, Cold Spring Harbor Laboratory, NY) were cotransfected using Fugene 6 with pBabe-Ha-rasV12 and pShh-p53 (no resistance marker), according to the manufacturer's recommendations. Selection was for the presence of the activated *HarasV12* plasmid, which carries a puromycin-resistance marker. The pShh-p53 plasmid was present in excess, as is standard in a cotransfection experiment. We have now generated a version of the U6 promoter vector (pSHAG-1) that is compatible with the GATEWAY system (Invitrogen), and this can be used to transport the shRNA expression cassette into a variety of recipient vectors that carry *cis*-linked selectable markers. Furthermore, we have validated delivery of shRNAs using retroviral vectors. Updated plasmid information can be obtained at http://www.cshl.org/public/science/hannon.html.

Plasmids expressing hairpin RNAs

The U6 promoter region from −265 to +1 was amplified by PCR, adding 5′ *Kpn*I and 3′ *Eco*RV sites for cloning into pBSSK+. A linker/terminator oligonucleotide set bearing the U6 terminator sequence and linker ends of 5′ *Eco*RV and 3′ *Not*I was cloned into the promoter construct, resulting in a U6 cassette with an *Eco*RV site for insertion of new sequences. This vector has been named pShh1. Blunt-ended, double-stranded DNA oligonucleotides encoding shRNAs with between 19 and 29 bases of homology to the targeted gene were ligated into the *Eco*RV site to produce expression constructs. The oligonucleotide sequence used to construct Ff1 was: TCCAATTCAGCGGGAGCCACC TGATGAAGCTTGATCGGGTGGCTCTCGCTGAGTTGGA ATCCATTTTTTTT. This sequence is preceded by the sequence GGAT, which is supplied by the vector, and contains a tract of more than five Ts as a Pol III terminator.

In vitro Dicer assays

In vitro assays for Dicer activity were performed as described (Bernstein et al. 2001a).

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Table 1 Nodal cilia features in different vertebrate classes

	Onset of Lrdr mRNA expression	*Appearance of nodal cilia*	*Earliest conserved asymmetric gene expression*
Mouse	Gastrula, 7.5 d.p.f.	Gastrula, 7.5 d.p.f.	*Nodal*, 8.25 d.p.f., 3–5 somites
Chick	Gastrula, HH4 –	Gastrula, HH4 –	*Nodal*, HH7*, 0–2 somites
Xenopus	Stage-11 gastrula	Stage-14 neurula	*Xnr-1* (*Nodal*), stage-17 neurula
Zebrafish	80% epiboly gastrula	Four somites	*Cyclops* (*Nodal*), 20 somites

*Asymmetric gene expression of *Sonic hedgehog* at the node occurs at HH5 (gastrulation) before *Nodal* expression, but seems to be unique to the chick embryo. d.p.f., days post-fertilization.

localization of *Lrdr* expression and formation of nodal cilia. The earliest known asymmetric expression patterns that are common to all vertebrates likewise exhibit considerable variability in their time of onset among different vertebrate classes[1,2]. In all instances, however, these conserved asymmetries are preceded by the onset of *Lrdr* expression and by the appearance of nodal cilia (Table 1), indicating that nodal cilia may be responsible for initiating L–R asymmetric gene expression and for establishing the final body plan in all vertebrates.

Jeffrey J. Essner*, Kyle J. Vogan†, Molly K. Wagner*, Clifford J. Tabin†, H. Joseph Yost*, Martina Brueckner‡

*Center for Children, Huntsman Cancer Institute and Department of Oncological Sciences, University of Utah, Salt Lake City, Utah 84112-5550, USA
e-mail: joseph.yost@hci.utah.edu
†Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA
‡Department of Pediatrics and Cardiology, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, USA

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Gene expression

RNA interference in adult mice

RNA interference is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. Here we show that transgene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed *in vivo* from DNA templates. We also show the therapeutic potential of this technique by demonstrating effective targeting of a sequence from hepatitis C virus by RNA interference *in vivo*.

Small interfering RNAs (siRNAs) mimic intermediates in the RNA-interference (RNAi) pathway and can silence genes in somatic cells without activating non-specific suppression by double-stranded RNA-dependent protein kinase[1]. To investigate whether siRNAs also inhibit gene expression *in vivo*, we used a modification of hydrodynamic transfection methods[2–4] to deliver naked siRNAs to the livers of adult mice. Either an siRNA derived from firefly luciferase or an unrelated siRNA was co-injected with a luciferase-expression plasmid (for construct description and sequences, see supplementary information). We monitored luciferase expression in living animals using quantitative whole-body imaging[5] (Fig. 1a, c), and found that it was dependent on reporter-plasmid dose (results not shown).

In each experiment, serum measurements of a co-injected human α-1 antitrypsin (hAAT) plasmid[6] served to normalize transfection efficiency and to monitor non-specific translational inhibition. Average serum concentrations of hAAT after 74 h were similar in all groups.

Our results indicate that there was specific, siRNA-mediated inhibition of luciferase expression in adult mice ($P < 0.0115$) and that unrelated siRNAs had no effect ($P < 0.864$; Fig. 1a, b). In 11 independent experiments, luciferase siRNAs reduced luciferase expression (as judged by emitted light) by an average of 81% (±2.2%). These findings indicate that RNAi can downregulate gene expression in adult mice.

As RNAi degrades respiratory syncitial virus RNAs in culture[7], we investigated whether RNAi could be directed against a human pathogenic RNA expressed in a mouse, namely that of hepatitis C virus (HCV). (Infection by HCV, an RNA virus that infects 1 in 40 people worldwide, is the most common reason for liver transplantation in the United States and Europe.) We fused the NS5B region (non-structural protein 5B, viral-polymerase-encoding region) of this virus with luciferase RNA and monitored RNAi by co-transfection *in vivo*. An

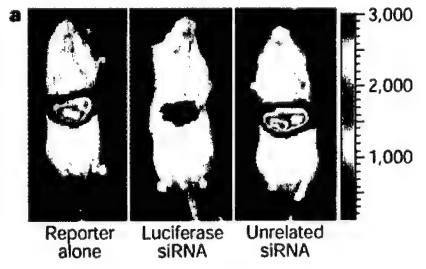

Figure 1 RNA interference in adult mice. **a,** Representative images of light emitted from mice co-transfected with the luciferase plasmid pGL3-control and either no siRNA, luciferase siRNA or unrelated siRNA. A pseudocolour image representing intensity of emitted light (red, most intense; blue, least intense) superimposed on a greyscale reference image (for orientation) shows that RNAi functions in adult mice. Annealed 21-nucleotide siRNAs (40 μg; Dharmacon) were co-injected into the livers of mice with 2 μg pGL3-control DNA (Promega) and 800 units of RNasin (Promega) in 1.8 ml PBS buffer in 5–7 s. After 72 h, mice were anaesthetized and given 3 mg luciferin intraperitoneally 15 min before imaging. **b,** siRNA results (six mice per group) from a representative experiment. Mice receiving luciferase siRNA emitted significantly less light than reporter-alone controls (one-way ANOVA with post hoc Fisher's test). Results for reporter alone and unrelated siRNA were statistically similar. **c,** pShh1-Ff1, but not pShh1-Ff1rev (see text), reduced luciferase expression in mice relative to the reporter-alone control. pShh1-Ff1 or pShh1-rev (10 μg) were co-injected with 2 μg pGL3-control in 1.8 ml PBS buffer. **d,** Average of three independent shRNA experiments ($n = 5$). Average values for the reporter-alone group are designated as 100% in each of the three experiments. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.

Appendix 2

siRNA targeting the NS5B region reduced luciferase expression from the chimaeric HCV NS5B protein–luciferase fusion by 75% (±6.8%; 6 animals per group). This result suggests that it may be feasible to use RNAi as a therapy against other important human pathogens.

Although our results show that siRNAs are functional in mice, delivery remains a major obstacle. Unlike siRNAs, functional small-hairpin RNAs (shRNAs) can be expressed *in vivo* from DNA templates using RNA polymerase III promoters[8,9]; they are as effective as siRNAs in inducing gene suppression. Expression of a cognate shRNA (pShh1-Ff1; see supplementary information) inhibited luciferase expression by up to 98% (±0.6%), with an average suppression of 92.8% (±3.39%) in three independent experiments (Fig. 1c, d). An empty shRNA-expression vector had no effect (results not shown); reversing the orientation of the shRNA (pShh1-Ff1rev) insert prevents gene silencing because it alters the termination by RNA polymerase III and generates an improperly structured shRNA. These findings indicate that plasmid-encoded shRNAs can induce a potent and specific RNAi response in adult mice.

RNAi may find application in functional genomics or in identifying targets for designer drugs. It is a more promising system than gene-knockout mice because groups of genes can be simultaneously rendered ineffective without the need for time-consuming crosses. Gene therapy currently depends on the ectopic expression of exogenous proteins; however, RNAi may eventually complement this gain-of-function approach by silencing disease-related genes with DNA constructs that direct the expression of shRNAs. Our method of RNAi delivery could also be tailored to take advantage of developing viral and non-viral gene-transfer vectors in a clinical context.

Anton P. McCaffrey*, Leonard Meuse*, Thu-Thao T. Pham*, Douglas S. Conklin†, Gregory J. Hannon†, Mark A. Kay*

**Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, California 94305-5208, USA*

e-mail: markay@stanford.edu

†Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA

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COMMUNICATIONS ARISING

Orbital physics

Experimental quest for orbital waves

One challenge in condensed-matter physics is the experimental confirmation of a new kind of elementary excitation — orbital waves, or orbitons, which are predicted to exist in an orbitally ordered state. Saitoh *et al.*[1] have observed three peaks at 160, 144 and 126 meV in the Raman scattering of orbitally ordered lanthanum manganate ($LaMnO_3$), and interpret these as evidence of orbitons. However, we find similar peaks in the optical conductivity, $\sigma(\omega)$, of $LaMnO_3$ and point out that the direct observation of orbitons in $\sigma(\omega)$ is prohibited by a selection rule. This suggests that the Raman peaks observed by Saitoh *et al.* arise from multiphonons, and that the existence of orbitons has yet to be experimentally confirmed.

We determined $\sigma(\omega)$ by measuring both the transmittance and reflectance of single crystals, using a sample polished to a thickness of $d \approx 62$ μm for the former. We compared $\sigma(\omega)$ of $LaMnO_3$ with the Raman data of Saitoh *et al.*[1] and found that the peaks in $\sigma(\omega)$ were similar to the Raman features (Fig. 1, top), albeit with slightly different frequencies (160, 146, 130 and 118 meV).

The orbital excitations discussed by Saitoh *et al.* involve transitions between orbital states of the same parity. These transitions do not contribute directly to $\sigma(\omega)$ owing to the parity selection rule; that is, they are not infrared-active. They may become weakly infrared-active in the presence of defects or by the simultaneous excitation of a parity-breaking Mn–O bond-stretching phonon[2]. The latter, phonon-activated mechanism is the more effective way to break the selection rule. Stronger features are therefore expected in $\sigma(\omega)$ at frequencies that — compared to the Raman peaks — are shifted by the respective phonon frequency of about 70 meV. As this is not the case, the peaks in $\sigma(\omega)$ cannot be explained by orbitons, challenging the orbiton interpretation of the Raman data.

We suggest that all peaks should be interpreted as arising from multiphonons. The sharp increase in $\sigma(\omega)$ at low frequen-

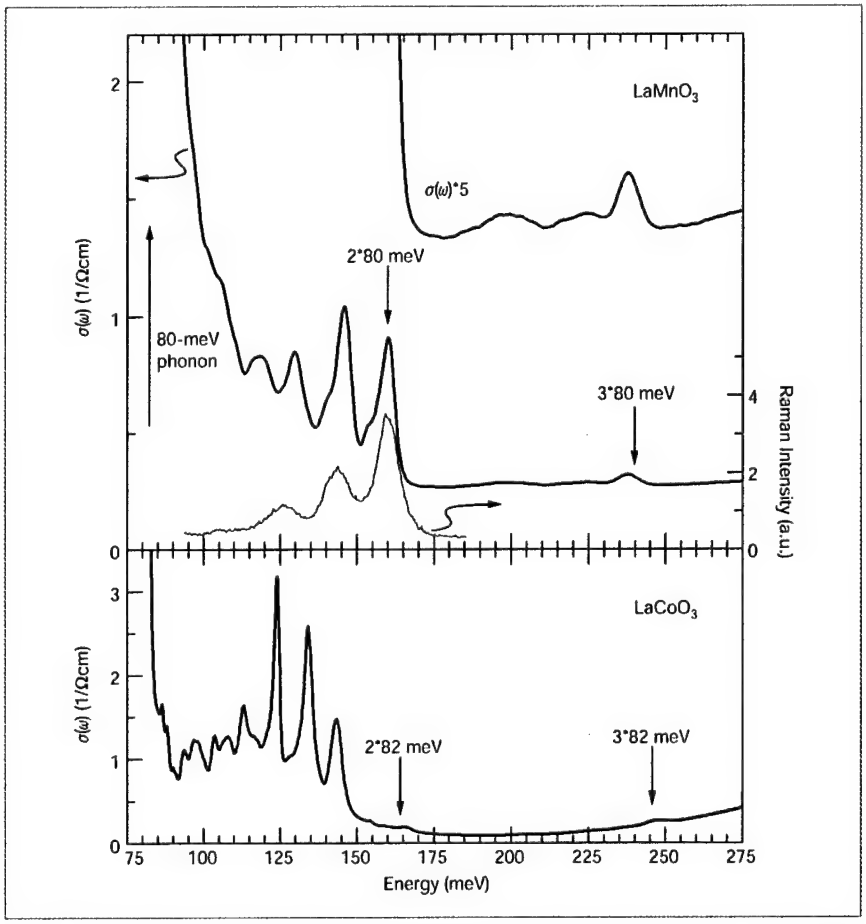

Figure 1 Optical conductivity $\sigma(\omega)$ of $LaMnO_3$ and $LaCoO_3$, and Raman measurements on $LaMnO_3$ at a temperature of 4 K. Top, comparison of $\sigma(\omega)$ (thick blue line) and Raman intensity (from Saitoh *et al.*[1]; thin yellow line) of $LaMnO_3$ reveals a close similarity between the two spectra. In $\sigma(\omega)$ the highest peaks of single-, two- and three-phonon absorption are observed at 80, 160 and 240 meV, respectively. Inset, enlargement of the three-phonon range of $\sigma(\omega)$. Bottom, $\sigma(\omega)$ of $LaCoO_3$, showing multiphonon peaks, for example at 2*82 and 3*82 meV.

An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes *in vivo*

Michael T. Hemann[1], Jordan S. Fridman[1], Jack T. Zilfou[1], Eva Hernando[2], Patrick J. Paddison[1], Carlos Cordon-Cardo[2], Gregory J. Hannon[1] & Scott W. Lowe[1]

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The application of RNA interference (RNAi) to mammalian systems has the potential to revolutionize genetics and produce novel therapies. Here we investigate whether RNAi applied to a well-characterized gene can stably suppress gene expression in hematopoietic stem cells and produce detectable phenotypes in mice. Deletion of the *Trp53* tumor suppressor gene greatly accelerates *Myc*-induced lymphomagenesis, resulting in highly disseminated disease[1,2]. To determine whether RNAi suppression of *Trp53* could produce a similar phenotype, we introduced several *Trp53* short hairpin RNAs (shRNAs) into hematopoietic stem cells derived from Eμ-Myc transgenic mice, and monitored tumor onset and overall pathology in lethally irradiated recipients. Different *Trp53* shRNAs produced distinct phenotypes *in vivo*, ranging from benign lymphoid hyperplasias to highly disseminated lymphomas that paralleled *Trp53*$^{-/-}$ lymphomagenesis in the Eμ-Myc mouse. In all cases, the severity and type of disease correlated with the extent to which specific shRNAs inhibited p53 activity. Therefore, RNAi can stably suppress gene expression in stem cells and reconstituted organs derived from those cells. In addition, intrinsic differences between individual shRNA expression vectors targeting the same gene can be used to create an 'epi-allelic series' for dissecting gene function *in vivo*.

RNAi is a powerful tool for manipulating gene expression in model organisms and cultured mammalian cells[3]. The technology arose from the observation that exogenous double-stranded RNAs induce gene silencing in plants and *Caenorhabditis elegans*. These double-stranded RNAs are processed into small interfering RNAs (siRNAs), which are incorporated into a conserved cellular machinery that mediates the suppression of homologous genes. Recently, small non-coding RNAs have been identified that can act as endogenous regulators of gene expression. These microRNAs typically form stem-loop structures, essentially short double-stranded RNAs, that enter the RNAi pathway[4–7]. shRNAs, modeled after microRNAs, can be expressed from viral vectors to induce stable suppression of gene expression in cultured mammalian cells[8].

We reasoned that stable suppression of gene expression by RNAi should recapitulate the phenotype of mice harboring deletions of the targeted gene. To test this, we targeted the *Trp53* tumor suppressor, a gene with extensively characterized loss-of-function

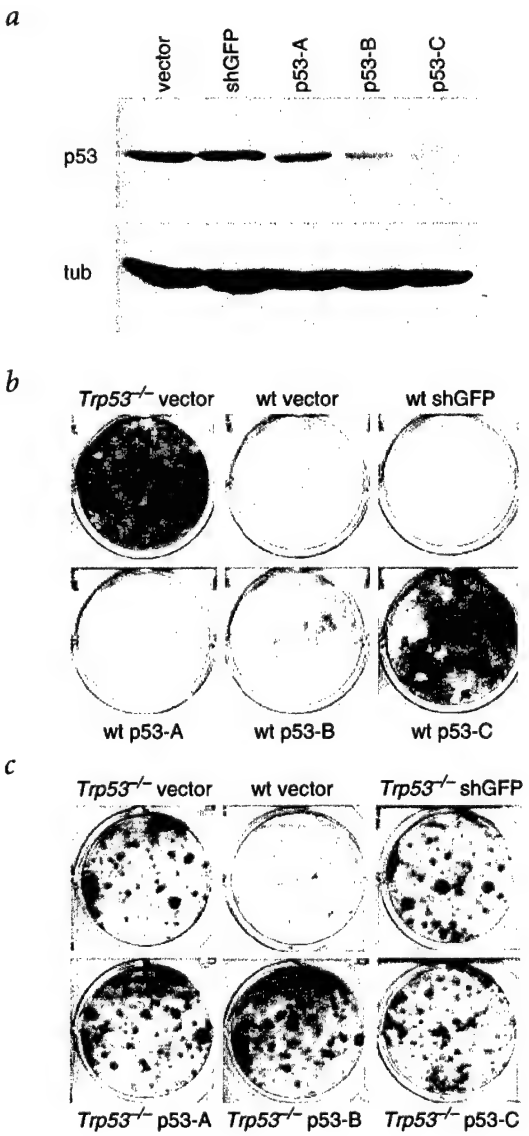

Fig. 1 Analysis of *Trp53* shRNA function *in vitro*. *a*, Western blot showing the lower p53 levels in H1299 cells transiently transfected with different *Trp53* shRNA retroviral vectors. Tubulin (tub) expression shows equal loading of all samples. *b*, Colony-formation assay on wild-type (wt) MEFs infected with an empty vector (vector), a vector expressing a nonspecific shRNA (shGFP) and vectors expressing the indicated *Trp53* shRNAs. *Trp53*$^{-/-}$ MEFs infected with an empty vector (vector) are shown as a control. A total of 5,000 cells were plated per well and cultured for 2 wk before staining with crystal violet. *c*, Colony-formation assay on *Trp53*$^{-/-}$ MEFs infected with control and p53 shRNAs. A total of 1,000 cells were plated per well and analyzed as in *b*. Wild-type (wt) MEFs infected with an empty vector (vector) are shown as a control.

[1]*Cold Spring Harbor Laboratory, Watson School of Biological Sciences, Cold Spring Harbor, New York 11724, USA.* [2]*Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York, USA. Correspondence should be addressed to G.J.H. (e-mail: hannon@cshl.org) or S.W.L. (e-mail: lowe@cshl.org).*

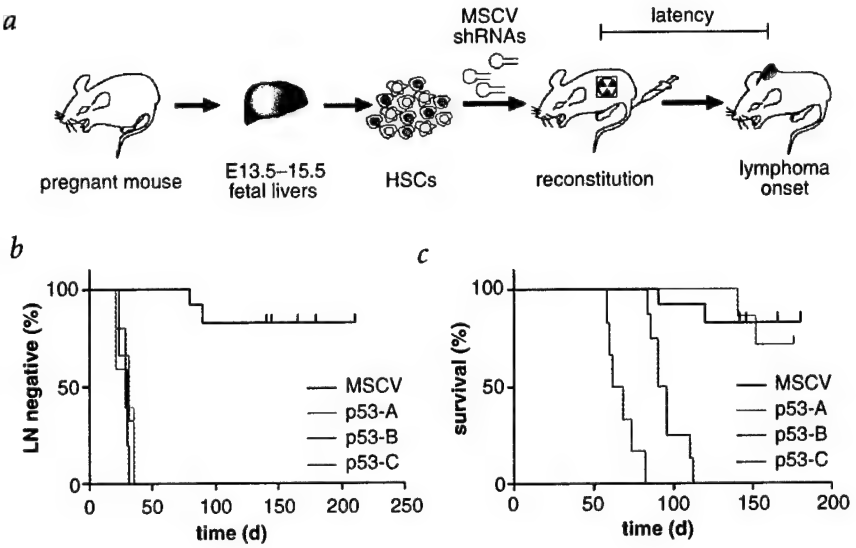

Fig. 2 Acceleration of Eμ-Myc–induced lymphomagenesis by *Trp53* shRNAs. ***a***, Protocol for adoptive transfer experiments using Eμ-Myc hematopoietic stem cells infected with shRNA vectors. ***b***, Mice reconstituted with stem cells infected with the indicated *Trp53* shRNAs were monitored for lymph node enlargement by regular palpation of the axillary, brachial and inguinal nodes. The data are presented in a Kaplan-Meier format showing the percentage of mice that remained free of enlarged lymph nodes at various times after reconstitution (time 0). All mice receiving *Trp53* shRNAs showed lymph node hyperplasia 3–5 wk after injection, whereas control vector recipients did not. ***c***, The animals described in *b* were monitored for tumor onset and illness until they reached a terminal stage and were killed. The data are presented in a Kaplan-Meier format showing overall survival at various times after reconstitution. The p53-B and p53-C, but not p53-A, constructs accelerate lymphomagenesis in recipient animals.

phenotypes[9]. The protein p53 encoded by *Trp53* promotes apoptosis in response to hyperproliferative signals; therefore, p53 inactivation accelerates tumorigenesis initiated by mitogenic oncogenes[10] . For example, Eμ-Myc transgenic mice express the *Myc* oncogene from an immunoglobulin heavy-chain enhancer and develop B-cell lymphomas by 4–6 months of age[11]. However, Eμ-Myc lymphomas harboring *Trp53* deletions arise much earlier, and display a characteristic disseminated pathology[1,2]. Hematopoietic stem cells from Eμ-Myc transgenic mice also give rise to lymphomas upon adoptive transfer into normal recipients, and these lymphomas are greatly accelerated by loss of *Trp53* or by anti-apoptotic genes introduced *ex vivo*[2]. Accordingly, stable suppression of p53 in Eμ-Myc hematopoietic stem cells by RNAi should accelerate lymphomagenesis in recipient animals.

We generated several retroviral vectors containing shRNAs targeting the tumor suppressor *Trp53*, designated p53-A, p53-B and p53-C (see Supplementary Fig. 1 online), and assessed their ability to influence p53 levels and activity. Transient transfection of these shRNA vectors into cultured cells resulted in varying levels of p53 suppression: p53-C achieved the greatest reduction in p53 levels, followed by p53-B and p53-A (Fig. 1*a*). Notably, a control GFP hairpin known to enter the RNAi pathway had no effect on p53 expression.

Colony-formation assays using mouse embryonic fibroblasts (MEFs) infected with these retroviruses also revealed differences among the *Trp53* shRNA vectors (Fig. 1*b*). In this assay, p53 deficiency results in a greatly enhanced ability of untransformed cells to form colonies when plated at clonogenic density. Consistent

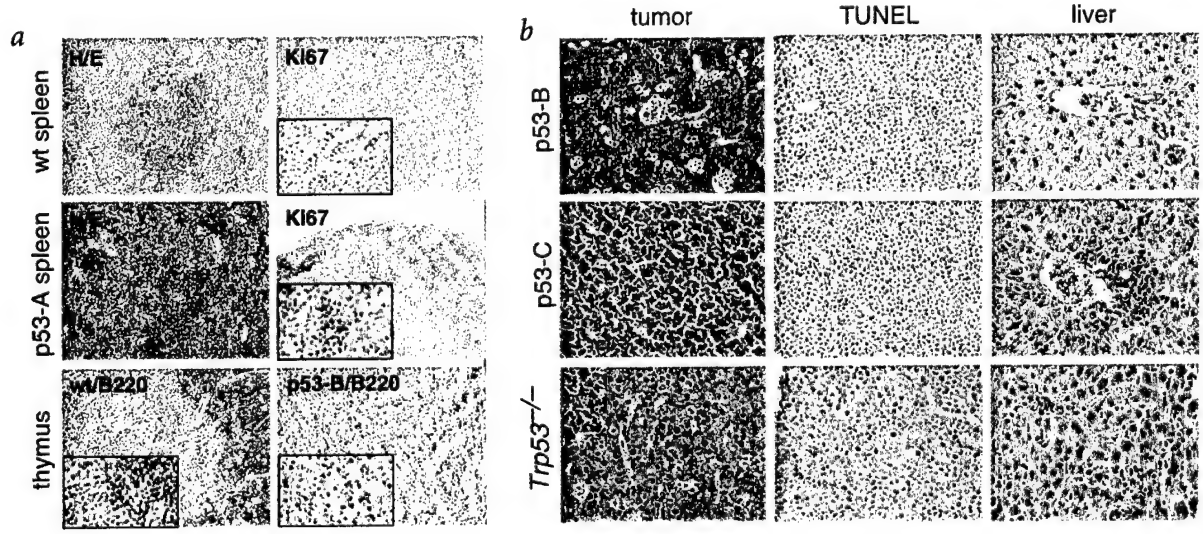

Fig. 3 *Trp53* shRNAs produce distinct pathologies *in vivo*. ***a***, Top, p53-A recipients show splenic hyperplasia. Spleen sections from a normal control and a p53-A recipient, isolated at day 50 after stem cell transplantation, are shown. The p53-A spleen shows a prominent white pulp and increased Ki67 staining (brown) relative to controls. Bottom, Mediastinal lymphomas in p53-B recipients compromise the normal thymic architecture. Whereas a wild-type (wt) thymus shows little B220 staining, the p53-B thymus is completely overtaken by B220-positive lymphoma cells. ***b***, H&E and TUNEL staining of p53-B, p53-C and *Trp53*$^{-/-}$ lymphoma and liver sections. H&E and TUNEL staining of lymphoma sections show clusters of apoptotic cells in p53-B tumors, whereas p53-C and *Trp53*$^{-/-}$ tumors show a relative lack of apoptotic cells. Liver sections from animals harboring p53-B lymphomas do not contain lymphoma cells, whereas livers from mice harboring p53-C and *Trp53*$^{-/-}$ lymphomas show perivascular and parenchymal infiltration of tumor cells.

with the varied abilities of the shRNAs to reduce p53 levels, p53-C produced the greatest colony formation, followed by p53-B and p53-A. None of the p53 shRNAs altered colony formation when expressed in *Trp53*$^{-/-}$ MEFs, indicating that these retroviruses probably have no off-target activities.

Viruses produced from the MSCV control and the p53-A, p53-B and p53-C retroviral vectors were used to infect hematopoietic stem cells isolated from Eμ-Myc fetal livers, and the infected cells were transplanted into lethally irradiated recipient mice (Fig. 2*a*). Whereas mice reconstituted with MSCV transduced (control) stem cells showed no initial signs of proliferative disease, mice reconstituted with stem cells expressing each *Trp53* shRNA vector had palpable lymph nodes 3–5 weeks after stem cell replacement (Fig. 2*b*). Despite this similarly timed onset of lymph node hyperplasia, the three *Trp53* shRNA vectors produced markedly different outcomes (Fig. 2*c*). Mice reconstituted with stem cells infected with p53-A, designated p53-A recipients, showed no decrease in overall survival relative to MSCV controls. In contrast, 8/8 p53-B and 6/6 p53-C recipients developed B-cell lymphomas and reached a terminal stage after an average of 95.0 ± 10.7 and 66.8 ± 9.4 days, respectively ($P < 0.001$ comparing MSCV controls to both p53-B and p53-C and $P < 0.05$ comparing p53-B to p53-C). Therefore, RNAi can suppress *Trp53* in hematopoietic stem cells, resulting in accelerated tumor phenotypes *in vivo*.

Pathological analysis showed marked differences between p53-A, p53-B and p53-C recipients. p53-A recipients showed lymph node enlargement, along with splenic hyperplasia as measured by Ki67 staining (Fig. 3*a*). But these mice did not develop lymphomas at an accelerated rate relative to MSCV controls ($P = 0.8$). The p53-B recipients developed small tumors throughout the lymphatic system and massive lymphomas surrounding and infiltrating the thymus, compromising the normal thymic architecture (Fig. 3*a*). These lymphomas could be transplanted into recipient animals, indicating that they were true malignancies and not severe hyperplasia (data not shown). The p53-B lymphomas showed high levels of apoptosis, a low mitotic index and only minor infiltration into the lung and liver (Fig. 3*b* and data not shown). In contrast, p53-C recipients were markedly similar to mice bearing *Trp53*-null lymphomas, showing only moderately enlarged mediastinal nodes but large disseminated lymphomas. In addition, p53-C tumors had low levels of apoptosis, a high mitotic index and massive lung, liver and spleen infiltration (Fig. 3*b* and data not shown). Therefore, different shRNAs targeting *Trp53* can elicit distinct phenotypes in recipient animals, such that the severity of the resulting tumor phenotype correlates with their ability to reduce p53 levels *in vitro*.

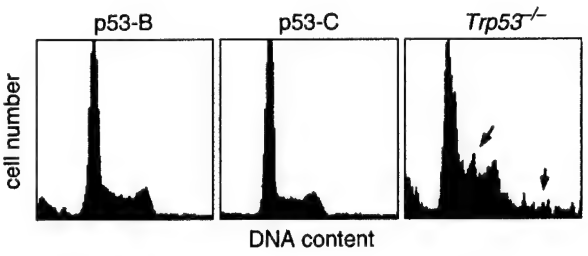

Fig. 4 *Trp53* shRNAs and chromosomal instability. p53-B and p53-C shRNAs lymphomas were compared to a *Trp53*$^{-/-}$ (p53-negative) lymphoma by DNA content analysis. The *Trp53*$^{-/-}$ lymphoma shows cells with abnormal DNA content (black arrows), whereas the tumors produced using *Trp53* shRNAs remain largely diploid. The results are representative of three tumors of each class.

Although lymphomas arising in p53-C shRNA recipients were similar to *Trp53*$^{-/-}$ lymphomas on pathological analysis, analysis of cellular DNA content showed substantial differences. As previously reported, *Trp53*$^{-/-}$ tumor cells were highly aneuploid[2]. In contrast, neither p53-B nor p53-C tumor cells had aberrant DNA content profiles (Fig. 4). These data indicate that the residual p53 levels in p53-B and p53-C tumor cells can preserve chromosome stability but cannot efficiently mediate apoptosis. Although further studies will be needed to determine precisely how different

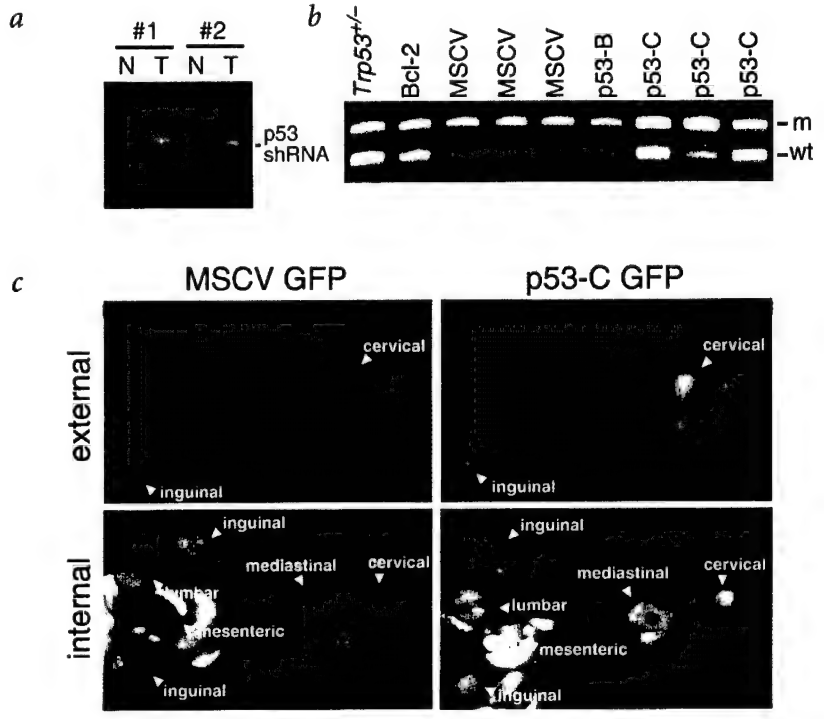

Fig. 5 *Trp53* shRNAs contribute to lymphomas and suppress mutations in endogenous *Trp53*. **a**, PCR analysis shows that the shRNA construct is present in tumor cells. Genomic DNA was isolated from normal (N) and tumor (T) cells from lymphoma-bearing mice and amplified using primers specific for the U6 promoter–hairpin cassette. Two examples are shown. **b**, *Trp53* shRNAs prevent LOH of *Trp53* in lymphomas arising from *Trp53*$^{+/-}$ cells. Normal DNA from a *Trp53*$^{+/-}$ mouse (left lane) and tumor DNA from mice harboring stem cells transduced with the indicated vector were subjected to allele-specific PCR to identify the mutant (m) and wild-type (wt) *Trp53* alleles. Owing to selection against p53 function, tumors derived from mice injected with *Trp53*$^{+/-}$ stem cells containing MSCV vector show LOH. In contrast, tumors derived from mice receiving stem cells containing *Trp53* shRNAs show retention of the wt allele. As previously shown[2], Bcl-2 also prevents LOH of *Trp53* in this setting. **c**, Detection of GFP in tumors arising from *Trp53*$^{+/-}$ stem cells containing a *Trp53* shRNA co-expressed with GFP. External and internal images of the ventral side of tumor-bearing mice are shown. GFP expression indicates the presence of the vector in the resulting lymphoma. Although an MSCV GFP control vector does not enhance lymphomagenesis in *Trp53*$^{+/-}$ cells, the p53-C GFP vector is present in all resulting lymphomas.

degrees of p53 suppression affect p53 effector functions, these results underscore the importance of the apoptotic activity of p53 in suppressing *Myc*-induced lymphomagenesis[2].

We used PCR analysis to confirm the presence of the hairpin construct in accelerated tumors. As expected, 6/6 primary p53-B and p53-C tumors tested contained the MSCV hairpin construct (Fig. 5*a* and data not shown). The constructs were also detected in transplanted tumors, eliminating the possibility that the amplified band arose from non-malignant stem cell–derived cells present in the primary tumors.

Although spontaneous *Trp53* mutations occur in only 10–15% of Eμ-Myc lymphomas, 100% of *Trp53*$^{+/-}$ Eμ-Myc mice develop tumors resulting from loss of heterozygosity (LOH) at the *Trp53* locus[1]. Because the selective pressure to undergo LOH in this context is so high, *Trp53*$^{+/-}$ Eμ-Myc stem cells can serve as an acutely sensitive system for testing whether *Trp53* shRNAs can impair p53 function. Given that *Trp53* shRNAs impair p53 function in other assays, we predicted that RNAi suppression of *Trp53* would allow a proportion of the final tumor mass derived from Eμ-Myc *Trp53*$^{+/-}$ stem cells to develop without LOH of *Trp53*. To test this prediction, we introduced MSCV control, p53-B and p53-C retroviral vectors into *Trp53*$^{+/-}$ Eμ-Myc hematopoietic stem cells and allowed lymphomas to form after adoptive transfer into recipient mice. As previously shown, all tumors derived from *Trp53*$^{+/-}$ stem cells infected with the MSCV vector showed LOH on allele-specific PCR (Fig. 5*b*). In contrast, 1/2 tumors derived from *Trp53*$^{+/-}$ cells containing p53-B and 4/4 tumors derived from *Trp53*$^{+/-}$ cells containing p53-C developed tumors that retained the wild-type *Trp53* allele (Fig. 5*b* and data not shown). Thus, LOH is not necessary for tumor development in the presence of a *Trp53* shRNA.

We visualized *in vivo* the contribution of *Trp53* shRNAs to tumorigenesis by introducing p53-C using a retroviral vector that co-expressed GFP (Fig. 5*c*), which allowed tracking of infected cells by whole-body imaging[2]. Tumors arising in mice that had received *Trp53*$^{+/-}$ Eμ-Myc stem cells infected with this p53-C GFP vector were GFP positive and retained the wild-type *Trp53* allele (Fig. 5*c* and data not shown), whereas lymphomas arising from stem cells infected with MSCV GFP alone were GFP negative and had lost the wild-type *Trp53* allele. Thus, *Trp53* shRNAs promote tumor development by abrogating the requirement for *Trp53*$^{+/-}$ cells to delete the wild-type *Trp53* allele during lymphomagenesis.

Our results have broad implications for mammalian genetics and the use of RNAi as a therapeutic tool. First, they establish stable RNAi as an alternative to homologous recombination for producing loss-of-function phenotypes in mice. Previously, such analyses required the generation of germline mutations. Although homologous recombination remains the only technique for producing complete gene deficiencies, combining stable RNAi with stem cell–based reconstitution of target organs is much more rapid and circumvents the complications that arise from the presence of nullizygous mutations during embryogenesis. Second, our results indicate that shRNAs with intrinsically different abilities to suppress a target gene *in vitro* conserve these relative biological activities *in vivo*. This permits the construction of an epi-allelic series of hypomorphic mutations that, in the case of the *Trp53* shRNAs, produces distinct types of hyperplastic diseases based on differing strengths of p53 suppression. Tumor suppressors often act as components of complex networks, the overall function of which can be impaired by many different genetic or epigenetic alterations. The ability to dampen such networks to different degrees will be of enormous value in studying early stages of disease, which are neither easily nor quickly recapitulated using germline genetic alterations. Finally, our studies provide strong support for the notion that stable RNAi suppression of deleterious genes might be used in therapeutic regimens for human diseases in which stem cells are modified *ex vivo* and then re-introduced into the affected individual.

Methods

Retroviral vectors. We generated retroviruses encoding shRNAs expressed from the U6 promoter by PCR using a pGEM U6 promoter template as previously described[8]. The amplification reactions used a universal 5′ primer corresponding to the SP6 site at the 5′ end of the U6 promoter cassette and a long 3′ primer complementary to the 3′ end of the promoter followed by sequences encoding the *Trp53* shRNA and a pol III termination site. We designed the shRNA sequences using designated software found at the RNAi OligoRetriever Database and encoded inverted repeats of 27–29 bp separated by an 8-nt spacer. The inverted repeats corresponded to nt 266–293 (shp53.1) or 44–72 (shp53.2) of the mouse *Trp53* cDNA, and had at least 3-nt differences from any other murine genes as determined by BLAST. We cloned the resulting PCR products directly into a pENTR/TOPO-D vector (Invitrogen) and then transferred the U6-shRNA cassette to one of several retroviral vectors containing a 'Gateway destination cassette' (Invitrogen) in either the *Nhe*I site of pBabePuro or the *Hpa*I site of MSCV. Altogether, we produced the following retroviral vectors: pBabe p53-A, encoding the shp53.1 hairpin in the 3′ LTR of pBabePuro[8]; pMSCV p53-B, encoding the shp53.1 hairpin 5′ of the PGK-Puro construct encoded by pMSCVpuro (Clontech); pMSCV p53-C, encoding the shp53.2 hairpin 5′ of the PGK-Puro construct encoded by pMSCVpuro; pMSCV GFP, encoding IRES-GFP 3′ of the PGK-Puro construct encoded by pMSCVpuro; and pMSCV p53-C GFP, encoding the shp53.2 hairpin 5′ of the PGK-Puro-IRES-GFP encoded by pMSCV GFP. Cloning strategies and primer sequences are available from the authors on request.

Suppression of p53 levels and activity by *Trp53* shRNAs. We co-transfected H1299 human lung adenocarcinoma cells with plasmids encoding *Trp53* and different *Trp53* shRNAs at a 1:5 ratio using the Fugene reagent (Roche); 36–48 h later, we extracted cells in RIPA buffer (50 mM Tris pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; and 1% sodium deoxycholate) supplemented with Complete Mini protease inhibitors (Roche). We assessed p53 expression by immunoblotting using 50 μg of total cell lysate and an enhanced chemiluminescence (ECL) detection system as described[12], with primary antibodies directed against p53 (1:1,000; CM5, NovoCastra) or α-tubulin (1:2,000; B-5-1-2, Sigma).

We assessed the impact of various *Trp53* shRNAs on p53 function and 'off-target' activities using a colony-formation assay in MEFs. We obtained wild-type and *Trp53*$^{-/-}$ MEFs from embryonic day 13.5 (E13.5) embryos and infected these cells with retroviruses co-expressing various shRNAs and a puromycin-resistance marker (*puro*) as described[13]. We incubated the resulting cell populations for 48 h in the presence of 2 μg/ml puromycin and then plated them at low density in 6-well plates. Two weeks later, the colonies were visualized by crystal violet staining.

RNAi of *Trp53* in stem cells. We isolated, infected and transplanted hematopoietic stem cells exactly as described[2]. We crossbred Eμ-Myc transgenic mice[11] to wild-type (*Trp53*$^{+/+}$) or *Trp53*$^{+/-}$ C57BL/6 mice (Jackson Laboratory) and obtained hematopoietic stem cells from E13.5–15.5 fetal livers. We maintained all mice in the C57BL/6 background and confirmed all stem cell genotypes by PCR. We cultured Eμ-Myc *Trp53*$^{+/+}$ and Eμ-Myc *Trp53*$^{+/-}$ fetal liver cells under conditions that support stem cell proliferation and infected the cells with various shRNA-expressing retroviruses exactly as described[2]. Under these conditions we typically infected 5–15% of fetal liver cells. Then, 36 h later, we used 1×10^6 cells to reconstitute the hematopoietic compartment of lethally irradiated mice (approximately 9 Gray per mouse). The Cold Spring Harbor Animal Care and Use Committee approved all mouse experiments included in this work.

Lymphoma monitoring and analysis. We monitored reconstituted animals for illness by lymph node palpation, by monitoring overall morbidity and, in some cases, by whole-body fluorescence imaging[2]. We assessed the onset of lymph node hyperplasia by regular palpation of the axillary, brachial and inguinal lymph nodes. We defined overall survival as the time from stem cell reconstitution until the animal reached a terminal stage and

had to be killed. In all cases, terminal animals harbored large tumor burdens. We did statistical analysis using a one-way ANOVA test using Graph Pad Prism version 3.0 (Graph Pad Software).

We confirmed the presence of the *Trp53* hairpin in Eμ-Myc lymphomas by PCR amplification of lymphoma DNA with the primers used in generating the U6 promoter–shRNA constructs. We used DNA derived from the infected stem cell populations of the same mouse as a normal control. In experiments using MSCV GFP (control) or MSCV p53-C GFP retroviruses, we used whole-body fluorescence imaging of GFP to monitor the presence of transduced cells in the resulting lymphomas. We carried out external imaging on live mice, and we carried out internal imaging immediately after mice were killed. We determined the occurrence of LOH at the *Trp53* locus in lymphomas derived from $Trp53^{+/-}$ cells by allele-specific PCR of tumor DNA to identify the mutant (targeted) and wild-type *Trp53* alleles[2].

Histopathology. We fixed tissue samples in 10% buffered formalin and embedded them in paraffin, then stained sections (5 μm) with hematoxylin and eosin (H&E) according to standard protocols. For detection of p53 and Ki67, we deparaffinized, rehydrated and processed representative sections in graded alcohols using the avidin–biotin immunoperoxidase method. Briefly, we submitted sections to antigen retrieval by microwave oven treatment for 15 m in 10 mM sodium citrate buffer (pH 6.0). We incubated slides first in 10% normal goat serum for 30 m and then overnight at 4 °C with appropriately diluted primary antibody (rabbit antibody CM5 against mouse p53 and rabbit antibody against Ki67, both from NovoCastra). We next incubated the slides with biotinylated goat rabbit-specific immunoglobulins (Vector Laboratories) at 1:500 dilution for 30 m and then with avidin–biotin peroxidase complexes (1:25; Vector Laboratories) for 30 m. We used diaminobenzidine as the chromogen and hematoxylin as the nuclear counterstain. For B220 immunohistochemistry (rat antibody against mouse CD45R/B220–clone RA3-6B2, BD Biosciences, Pharmingen), antigen retrieval was required, and we used a biotinylated antibody against rat as a secondary antibody. We analyzed the apoptotic rate by TUNEL assay according to published protocols[14]. We determined the DNA content of tumor cells by FACS analysis with propidium iodide staining of ethanol-fixed cells as described[2].

URL. Design of shRNA primers from gene accession numbers can be done at the RNAi OligoRetriever Database, which can be reached through G.J.H.'s website at http://www.cshl.edu/public/SCIENCE/hannon.html.

Note: Supplementary information is available on the Nature Genetics website.

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Germline transmission of RNAi in mice

Michelle A. Carmell[1,2], Liqun Zhang[3], Douglas S. Conklin[1], Gregory J. Hannon[1] and Thomas A. Rosenquist[3,4]

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MicroRNA molecules (miRNAs) are small, noncoding RNA molecules that have been found in a diverse array of eukaryotes, including mammals. miRNA precursors share a characteristic secondary structure, forming short 'hairpin' RNAs. Genetic and biochemical studies have indicated that miRNAs are processed to their mature forms by Dicer, an RNAse III family nuclease, and function through RNA-mediated interference (RNAi) and related pathways to regulate the expression of target genes (reviewed in refs. 1,2). Recently, we and others (reviewed in ref. 3) have remodeled miRNAs to permit experimental manipulation of gene expression in mammalian cells and have dubbed these synthetic silencing triggers 'short hairpin RNAs' (shRNAs). Silencing by shRNAs requires the RNAi machinery and correlates with the production of small interfering RNAs (siRNAs), which are a signature of RNAi.

Expression of shRNAs can elicit either transient or stable silencing, depending upon whether the expression cassette is integrated into the genome of the recipient cultured cell (reviewed in ref. 3). shRNA expression vectors also induce gene silencing in adult mice following transient delivery[4,5]. However, for shRNAs to be a viable genetic tool in mice, stable manipulation of gene expression is essential. Hemann and colleagues[6] have demonstrated long-term suppression of gene expression *in vivo* following retroviral delivery of shRNA-expression cassettes to hematopoietic stem cells. Here we sought to test whether shRNA-expression cassettes that were passed through the mouse germline could enforce heritable gene silencing.

We began by taking standard transgenesis approaches[7] using shRNAs directed against a variety of targets with expected phenotypes, including the genes encoding tyrosinase (albino), myosin VIIa (shaker), Bmp-5 (crinkled ears), Hox a-10 (limb defects), homogentisate 1,2,-dioxygenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow). Three constructs per gene were linearized and injected into pronuclei to produce transgenic founder animals. Although we noted the presence of the transgene in some animals, virtually none showed a distinct or reproducible phenotype that was expected for a hypomorphic allele of the targeted gene.

Therefore, we decided to take another approach: verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking whether those cells retained suppression in a chimeric animal *in vivo*. We also planned to test whether such cells could pass a functional RNAi-inducing construct through the mouse germline. For these studies, we chose to examine a novel gene, *Neil1*, which is proposed to have a role in DNA repair. Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging and tissue damage following ischemia[8,9]. Oxidative DNA damage includes abasic sites, strand breaks and at least 20 oxidized bases, many of which are cytotoxic or pro-mutagenic[10]. DNA *N*-glycosylases initiate the base excision repair pathway by recognizing specific bases in DNA and cleaving the sugar base bond to release the damaged base[11].

The *Neil* genes are a newly discovered family of mammalian DNA *N*-glycosylases related to the Fpg/Nei family of proteins from *Escherichia coli*[12,13] (T.A.R., E. Zaika, A.S. Fernandes, D.O. Zharkov, H. Miller and A.P. Grollman, submitted). Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines from DNA, including thymine glycol (Tg), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formidopyrimidine (FapyA)[12,13] (T.A.R., E. Zaika, A.S. Fernandes, D.O. Zharkov, H. Miller and A.P. Grollman, submitted). Tg, FapyG and FapyA are among the most prevalent oxidized bases produced by ionizing radiation[10] and can block replicative DNA polymerases, which can, in turn, cause cell death[14,15].

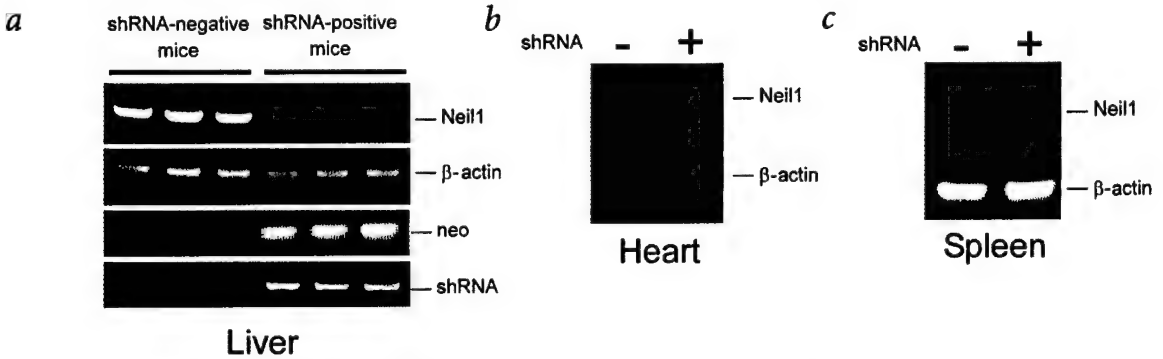

Fig. 1 Heritable repression of Neil1 expression by RNAi in several tissues. ***a***, Expression of *Neil1* mRNA in the livers of three mice containing the *Neil1* shRNA transgene (shRNA-positive) or three siblings lacking the transgene (shRNA-negative) was assayed by RT-PCR (top row is Neil1). An RT-PCR of β-actin was done to ensure that equal quantities of mRNAs were tested for each mouse (second row). Expression of the neomycin resistance gene (neo), carried on the shRNA vector, was tested similarly (third row). Finally, the mice were genotyped using genomic DNA that was PCR-amplified with vector-specific primers (bottom row). Similar studies were performed in the ***b***, heart and ***c***, spleen. Animal procedures have been approved by the SUNY, Stony Brook Institutional Animal Care and Use Committee (IACUC).

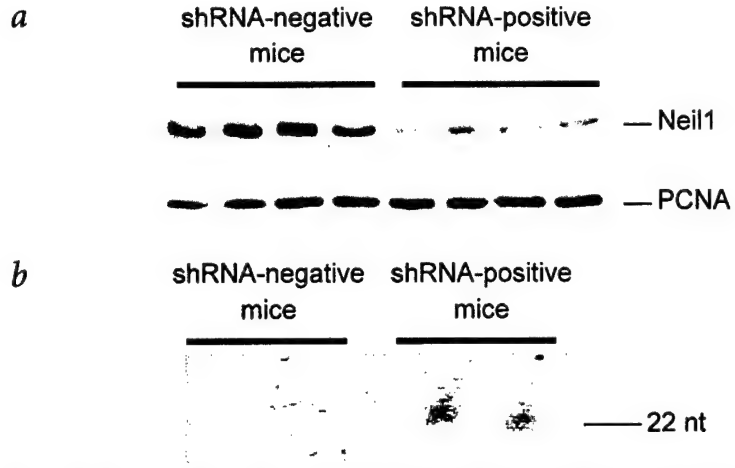

Fig. 2 Reduction in Neil1 protein correlates with the presence of siRNAs. ***a***, Expression of Neil1 protein was examined in protein extracts from the livers of mice carrying the shRNA transgene (shRNA-positive) or siblings lacking the transgene (shRNA-negative) by western blotting with Neil1-specific antiserum. A western blot for PCNA was used to standardize loading. ***b***, The presence of siRNAs in RNA derived from the livers of transgenic mice as assayed by northern blotting using a 300 nt probe, part of which was complementary to the shRNA sequence. We note siRNAs only in mice transgenic for the shRNA expression cassette.

The Nth1 and Ogg1 glycosylases each remove subsets of oxidized DNA bases that overlap with substrates of Neil1 (refs. 16–18). However, mice with null mutations in either *Nth1* (refs. 19,20) or *Ogg1* (refs. 21,22) are viable, raising the possibility that Neil1 activity tempers the loss of Nth1 or Ogg1. Recently, a residual Tg-DNA glycosylase activity in *Nth1*$^{-/-}$ mice has been identified as Neil1 (ref. 23).

We constructed a single shRNA expression vector targeting a sequence near the 5′ end of the *Neil1* coding region. This vector was introduced into mouse embryonic stem cells by electroporation, and individual stable integrants were tested for expression of the Neil1 protein (detailed procedures are available at *http://www.cshl.edu/public/SCIENCE/hannon.html*). The majority of cell lines showed an ~80% reduction in Neil1 protein, which correlated with a similar change in levels of *Neil1* mRNA (data not shown). These cells showed an approximately two-fold increase in their sensitivity to ionizing radiation (T.A.R., E. Zaika, A. S. Fernandes, D. O. Zharkov, H. Miller and A. P. Grollman, submitted), consistent with a role for Neil1 in DNA repair. Two independent ES cell lines were injected into BL/6 blastocysts, and several high-percentage chimeras were obtained. These chimeras were out-crossed, and germline transmission of the shRNA-expression construct was noted in numerous F_1 progeny (13/27 for one line and 12/26 for the other).

To determine whether the silencing of *Neil1* that had been observed in ES cells was transmitted faithfully, we examined *Neil1* mRNA and protein levels. Both were reduced by approximately the same extent that had been observed in the engineered ES cells (Figs. 1,2). Consistent with this having occurred through the RNAi pathway, we detected the presence of siRNAs corresponding to the shRNA sequence in F_1 animals that carry the shRNA expression vector but not in those that lack the vector (Fig. 2*b*).

The aforementioned data demonstrate that shRNAs can be used to create germline transgenic mice in which RNAi has silenced a target gene. These observations open the door to using of RNAi as a complement to standard knock-out methodologies and provide a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. Coupled with activator-dependent U6 promoters (P. Paddison, J. Du, E. Julien, W. Herr and G.J.H., unpublished data), the use of shRNAs will ultimately provide methods for tissue-specific, inducible and reversible suppression of gene expression in mice.

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[1]*Cold Spring Harbor Laboratory, Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA.* [2]*Program in Genetics, State University of New York at Stony Brook, Stony Brook, New York 11794, USA.* [3]*University Transgenic Mouse Facility, State University of New York, Stony Brook, New York 11794, USA.* [4]*Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794, USA.*

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Correspondence should be addressed to T.A.R.
e-mail: rosenquist@pharm.sunysb.edu

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RESEARCH COMMUNICATION

Fragile X-related protein and VIG associate with the RNA interference machinery

Amy A. Caudy, Mike Myers, Gregory J. Hannon,[1] and Scott M. Hammond[2]

Cold Spring Harbor Laboratory, Watson School of Biological Sciences, Cold Spring Harbor, NY 11724, USA

RNA interference (RNAi) is a flexible gene silencing mechanism that responds to double-stranded RNA by suppressing homologous genes. Here, we report the characterization of RNAi effector complexes (RISCs) that contain small interfering RNAs and microRNAs (miRNAs). We identify two putative RNA-binding proteins, the *Drosophila* homolog of the fragile X mental retardation protein (FMRP), dFXR, and VIG (Vasa intronic gene), through their association with RISC. FMRP, the product of the human fragile X locus, regulates the expression of numerous mRNAs via an unknown mechanism. The possibility that dFXR, and potentially FMRP, use, at least in part, an RNAi-related mechanism for target recognition suggests a potentially important link between RNAi and human disease.

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RNA interference (RNAi) encompasses a suite of homology-dependent gene silencing mechanisms that are triggered by double-stranded RNA (dsRNA). RNAi is an evolutionarily conserved response, and mechanistically related processes exist in plants, animals, and fungi (Bernstein et al. 2001b). A combination of genetic approaches in *Caenorhabditis elegans*, *Arabidopsis*, *Neurospora*, and *Chlamydomonas*, along with biochemical analyses in *Drosophila* extracts has begun to reveal a mechanistic framework for RNAi (Hammond et al. 2001b). Silencing is initiated when dsRNA triggers are processed into small interfering RNAs (siRNAs), which are ~21 nucleotides in length in *Drosophila* (Hamilton and Baulcombe 1999; Tuschl et al. 1999; Elbashir et al. 2001). This reaction is catalyzed by a group of related RNase III enzymes, now known as the Dicer family (Bernstein et al. 2001a). The siRNAs are incorporated into an effector complex, the RNA-induced silencing complex (RISC), which uses siRNAs as a guide to select complementary mRNA substrates (Hammond et al. 2000).

In general, RNAi acts at the posttranscriptional level and is associated with destabilization of mRNAs with complementarity to the silencing trigger (Fire et al. 1998). Recent studies suggest that some endogenously encoded small dsRNAs (known collectively as microRNAs, or miRNAs) can regulate the expression of endogenous protein-coding genes at the level of protein synthesis without message degradation (Olsen and Ambros 1999). The dependency of both siRNA- and miRNA-mediated silencing on Argonaute-family proteins and Dicer shows that the miRNA and siRNA pathways share conserved components and likely have related mechanisms. Recently, more than 100 miRNAs have been cloned from *C. elegans* (Lau et al. 2001; Lee and Ambros 2001), human (Lagos-Quintana et al. 2001; Mourelatos et al. 2002), mouse (Lagos-Quintana et al. 2002), *Drosophila* (Lagos-Quintana et al. 2001), and plants (Llave et al. 2002; Reinhart et al. 2002; A.A. Caudy, M. Myers, G.J. Hannon, and S.M. Hammond, unpubl.). Based on their prevalence, miRNAs are likely to play broad and important roles in gene regulation, possibly guiding complexes involved in various aspects of RNA metabolism to their cognate substrates. Both to address the underlying mechanism of siRNA-guided degradation and to investigate the degree to which the mechanisms of siRNA and miRNA-mediated silencing overlap, we have sought to determine the composition of RNAi-effector complexes.

Results and Discussion

Biochemical purification of the RNAi effector nuclease, RISC, revealed the *Drosophila* Argonaute-2 (Ago-2) protein as a core component of this complex (Hammond et al. 2001a). In an effort to identify additional RISC components, we performed large-scale biochemical purification, following both RISC activity and Ago-2 protein by Western blotting. After five purification steps, a number of additional proteins also consistently co-purified (Fig. 1A). Among those were two RNA-binding proteins, VIG and the *Drosophila* homolog of the human Fragile X Mental Retardation Protein [FMRP in human, dFXR in *Drosophila* (Wan et al. 2000); Fig. 1B,C]. Each protein was identified in multiple independent preparations of purified RISC.

The VIG protein is encoded from within an intron of the *Vasa* gene. This protein has no recognizable protein domains, other than an RGG box, a motif that is known to bind RNA. VIG is an evolutionarily conserved protein, with homologs in *C. elegans*, *Arabidopsis*, mammals, and *Schizosaccharomyces pombe*. Little is known regarding the function of this protein family. However, a human homolog of VIG, PAI-RBP-1, was originally identified as a protein with affinity for an AU-rich element in the 3′-UTR of the plasminogen activator inhibitor (PAI) RNA, which regulates its stability (Heaton et al. 2001).

A human ortholog of dFXR, FMRP, is encoded from a locus on the X chromosome that is epigenetically silenced in Fragile X syndrome (Eberhart et al. 1996). FMRP is expressed not only in neuronal cells but also in numerous other tissues, and Fragile X patients display additional phenotypes, including macroorchidism (Rudelli et al. 1983). The human genome also encodes two additional FMRP homologs, FXR1 and FXR2 (Kirkpatrick et al. 2001). Fragile X family members each contain two copies of a KH domain (hnRNP K homology) and an RGG box. All three of these domains have been proposed to bind RNA.

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Corresponding authors.
[1]E-MAIL hannon@cshl.org; FAX (516) 367-8874.
[2]E-MAIL hammond@cshl.org; FAX (516) 367-8874.
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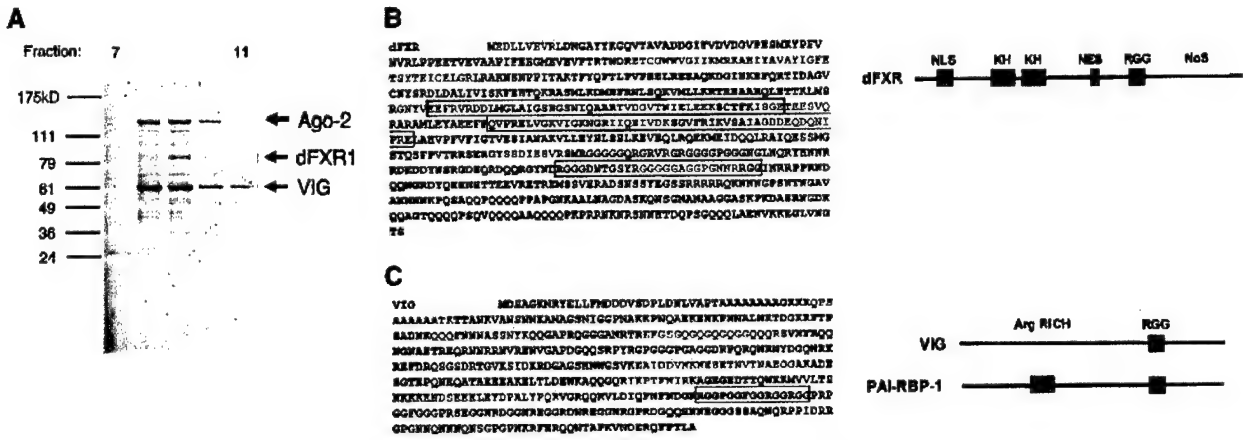

Figure 1. Identification of dFXR [the *Drosophila* homolog of the Fragile X Mental Retardation Protein (FMRP)], and VIG as RISC components. (*A*) Coomassie-stained polyacrylamide gel showing purified RISC fractions from a Source Q column. (*B*) Peptides obtained from microsequencing are indicated in red on the dFXR protein sequence. The KH (hnRNP K homology) domains are boxed in black and the RGG box is indicated in blue (accession no. dFXR NP_611645). (*C*) Peptides obtained from microsequencing are indicated in red on the VIG protein sequence; the RGG box is indicated in blue. At *right*, a comparison of the domain structure of VIG to the human homolog PAI-RBP-1 (accession nos. VIG NP_523572 and PAI-RBP-1 NP_056455).

To test the association of VIG and dFXR with RISCs, we expressed epitope-tagged proteins in *Drosophila* S2 cells. Notably, both Vig and dFXR co-fractionated with Ago-2 and RISC activity in an ~500-kD complex, as predicted if all three were components of RISC (Fig. 2A). Both RISC and dFXR pellet in a high-speed centrifugation and can be extracted from the P100 pellet by treatment with 0.4 M KOAc (Fig. 2B). Essentially, all expressed dFXR co-fractionated with RISC through subsequent ion exchange columns (Fig. 2C; data not shown). In accord with this hypothesis, immunoprecipitation of dFXR resulted in co-precipitation of Ago-2 protein (Fig. 2D). To test whether the interaction was bridged by binding separately to a common mRNA, we treated immunoprecipitations with RNAse A. Interaction between Ago-2 and dFXR was resistant to treatment of the immunoprecipitates with RNAse A up to concentrations of 10 mg/mL (data not shown). Similarly, Ago-2 was also recovered in immunoprecipitations of epitope-tagged VIG (Fig. 2E), and VIG and dFXR were present in mutual immunoprecipitations (data not shown).

The presence of VIG and dFXR in RISCs would predict that these proteins would also interact with siRNAs, another established component of RISCs (Hammond et al. 2000). To test this possibility, we co-transfected *Drosophila* S2 cells with ^{32}P-labeled dsRNA triggers of ~500 nt in length and either tagged VIG or dFXR. In each case, immunoprecipitation selectively recovered ~21 nt siRNAs, even though these are a minor component in analyses of total RNA extracted from cells transfected with labeled silencing triggers (Fig. 2E; data not shown).

To examine the requirement for dFXR and VIG in RNAi, we used RNAi to reduce the expression of each of these gene products in S2 cells. This approach has been used previously to test the dependency of RNAi on Dicer and Argonaute family members in *Drosophila* cells, mammalian cells, and *C. elegans* (Grishok et al. 2000; Bernstein et al. 2001a; Hammond et al. 2001a; Paddison et al. 2002; Tabara et al. 2002) and as a means to identify components of the RNAi machinery in worms (Dudley et al. 2002). Whereas cells pretreated with control dsRNAs (e.g. GFP) silence reporter genes efficiently, pretreatment of cells with dsRNAs covering the coding sequences of either dFXR or VIG partially impaired silencing. In the former case, expression returned to 50% of the unsuppressed control, whereas in the latter case, lucifer-

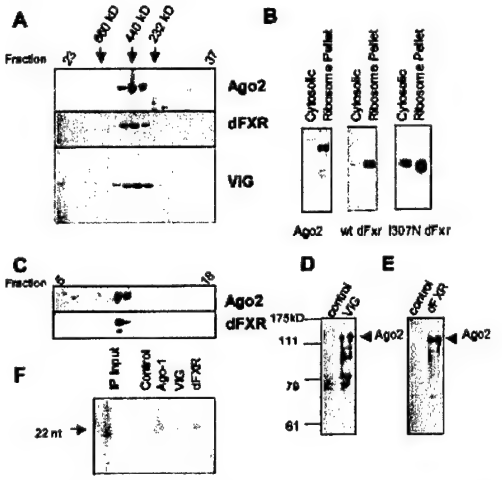

Figure 2. dFXR [the *Drosophila* homolog of the Fragile X Mental Retardation Protein (FMRP)] and VIG associate with RISC. (*A*) S2 cells were transfected with a combination of luciferase double-stranded RNA (dsRNA), his-tagged VIG, and T7-tagged dFXR1. Ribosomal extracts were size-fractionated on Superose 6 and Western blotted. (*B*) Western blots of Argonaute-2 (Ago-2) and tagged dFXR in cytoplasmic and high-salt ribosomal extract from cells expressing the indicated tagged constructs. (*C*) In a separate experiment, Flag-tagged dFXR-1 was transfected, extracted, and size-fractionated as in *A*, and fractions containing dFXR were further fractionated on Source S. Fractions were Western blotted for Ago-2 and Flag-dFXR. (*D*,*E*) *Drosophila* S2 cells were transfected with dsRNA and either a control vector or vectors directing the expression of either T7-VIG (*D*) or Flag-dFXR (*E*). Proteins were recovered by immunoprecipitation and analyzed by Western blot using a polyclonal antibody raised against the Ago-2 protein. (*F*) *Drosophila* S2 cells were transfected with radiolabeled luciferase dsRNA and the indicated control or T7-tagged expression constructs. Proteins were immunoprecipitated with anti-T7 affinity resin, and associated RNA was separated on denaturing 15% polyacrylamide gel.

ase was de-repressed to ~70% of the control (Fig. 3). The partial effect of suppressing dFXR can be interpreted in several ways. First, dFXR could be an accessory factor for RISCs, affecting efficiency of the complex but not being absolutely required for RNAi. Second, dFXR could be essential to only a subset of RISCs, whereas other, perhaps KH domain, proteins replace dFXR in alternative versions of RISCs. Third, dFXR might not be required for RNAi per se; however, loss of dFXR could affect the long-term stability of some RISC complexes. Another Argonaute family member expressed in S2 cells, Ago-1, appears to have little effect on the efficiency of RNAi in S2 cells. Ago-1 can be biochemically separated from active RISCs (data not shown). However, we by no means exclude a role for Ago-1 in RNAi (see below) as previous genetic studies (Williams and Rubin 2002) have indicated that Ago-1 is required for efficient RNAi during embryogenesis.

The forgoing biochemical and genetic studies are consistent with a model in which VIG and dFXR are associated with RISCs. We therefore tested the possibility that a sequence-specific nuclease activity might co-immunoprecipitate with either of these components. Cells were co-transfected with a dsRNA homologous to luciferase and with expression constructs for tagged versions of dFXR, VIG, or Ago-1. Control cells were transfected with a GFP expression construct. Immunoprecipitates were tested for RISC activity against substrates homologous to the dsRNA trigger and against nonhomologous substrates. Although immunoprecipitates from the GFP-transfected cells and immunoprecipitates of Ago-1 and Vig showed some specific activity against a synthetic luciferase mRNA, immunoprecipitates of dFXR substantially degraded the cognate but not the noncognate substrate. In the case of dFXR, >80% of the homologous substrate was degraded in 1 h of incubation (Fig. 4).

The majority of Fragle X mutations occur in the 5′-UTR of the gene and represent a trinucleotide repeat expansion that is thought to act by targeting FMR1 for epigenetic silencing. However, one point mutation in the FMRP protein itself has also been linked to the disease (I304N; De Boulle et al. 1993). This hypomorphic mutation in the human protein causes defects in ribosome association and RNA binding (Tamanini et al. 1996; Feng et al. 1997). We constructed an analogous

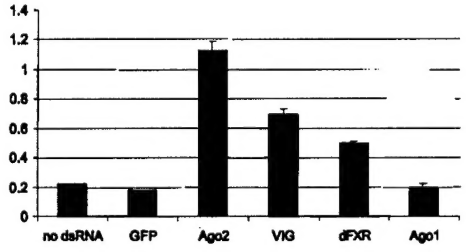

Figure 3. dFXR [the *Drosophila* homolog of the Fragile X Mental Retardation Protein (FMRP)] and VIG are required for efficient RNA interference in *Drosophila* S2 cells. *Drosophila* S2 cells were transfected with double-stranded RNAs (dsRNAs) corresponding to the indicated cDNAs. Three days later, cells were transfected with dsRNAs directed against luciferase (or GFP for controls) and with expression vectors for both firefly and renilla luciferases. Values shown represent the expression of luciferase in luciferase dsRNA-transfected cells relative to luciferase expression from cells transfected with control dsRNA.

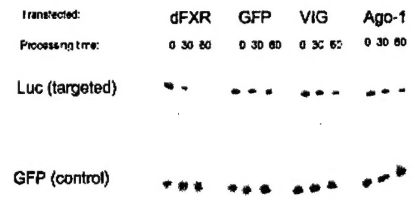

Figure 4. dFXR [the *Drosophila* homolog of the Fragile X Mental Retardation Protein (FMRP)] immunoprecipitates contain RISC activity. *Drosophila* S2 cells were transfected with expression vectors for the indicated T7-tagged cDNAs and double-stranded RNAs corresponding to luciferase. Protein was immunoprecipitated with anti-T7 tag affinity resin, and this resin was incubated for the indicated times with radiolabeled single-stranded RNA (ssRNA) for target (luciferase), or control (GFP). RISC activity was indicated by loss of targeted RNA.

mutation in the second KH domain of the *Drosophila* protein, I307N, and asked whether this mutation affected association with the RNAi machinery. Biochemical fractionation showed a significant alteration in the ability of the mutant dFXR to associate with RISC and Ago-2 (Fig. 2B), with ~30% of the mutant protein shifting from the ribosome pellet (where Ago-2 localizes) to a soluble cytoplasmic form. This fraction is completely devoid of Ago-2, and Ago-2 cannot be found in association with cytoplasmic dFXR.

The specific biochemical role of the human protein FMRP is not known; however, numerous studies suggest that it regulates the expression of a large number of genes at the level of protein synthesis (Bardoni and Mandel 2002). Recent studies of Dicer function (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) and of Argonaute family members (Grishok et al. 2001) in *C. elegans* have revealed that endogenously encoded small hairpin RNAs (generically, miRNAs) regulate the expression of protein coding genes via pathways related to RNAi. The presence of dFXR in RISCs suggests the hypothesis that FMRP or FMRP family members may regulate gene expression via a RISC-like complex.

Little is known of the effector complexes that contain miRNAs. A recent report has shown association of miRNAs in human cells with a 15S complex containing Gemin3, Gemin4, and eIF2c, a member of the Argonaute protein family (Mourelatos et al. 2002). Gemin3 is a DEAD-box family RNA helicase, and a helicase has previously been predicted as an essential activator of siRNA-containing complexes in *Drosophila* (Nykanen et al. 2001). Also, in *C. elegans* two DExH/D box helicases, one of which is Dicer, associate with Rde-4 and are required for RNAi (Tabara et al. 2002). Gemin4 lacks both recognizable protein motifs (Charroux et al. 2000) and an identifiable homolog in the *Drosophila* genome. Thus, the relationship between the previously characterized miRNP complex and RISC remains unclear, which led us to further characterize the association between miRNAs and RISCs.

A significant portion of two known miRNAs, mir2b and mir13a (Lagos-Quintana et al. 2001), in *Drosophila* S2 cells are found in association with polyribosomes, as would be predicted for small RNAs that regulate protein synthesis (data not shown). Furthermore, a functional connection between siRNAs and ribosome association

has been made in trypanosomes (E. Ullu, pers. comm.). As was previously shown for RISCs (Hammond et al. 2000), miRNAs can be extracted from a high-speed pellet from S2 cell extracts (which contains polysomes) using buffers containing elevated salt concentrations (Fig. 5A).

Extracted material was fractionated by size-exclusion chromatography, and the behavior of miRNAs and siRNAs was compared with that of known RISC subunits. As we previously reported (Hammond et al. 2000), the majority of siRNAs co-fractionate with Ago-2 and with VIG and dFXR. In contrast, the majority of miRNAs fractionate in an ~250-kD complex, although ~20% of miRNAs are present in fractions containing RISC (Fig. 5B). However, we could not rule out the possibility that this represents a tail of the 250-kD peak, given the nonquantitative nature of Western blotting. Considering the established role of dFXR in the regulation of endogenous protein-coding genes, we examined the possibility that miRNAs might be present in dFXR-containing complexes. Immunoprecipitates of dFXR were prepared from column fractions using appropriate affinity resins. Notably, mir2b was detected in dFXR immunoprecipitates but not in control immunoprecipitates prepared using the same affinity resins from Ago-2- and miRNA-containing fractions of extracts from untransfected cells (Fig. 5C). Similarly, VIG could be used to immunoprecipitate mir2B directly from extracts (Fig. 5D).

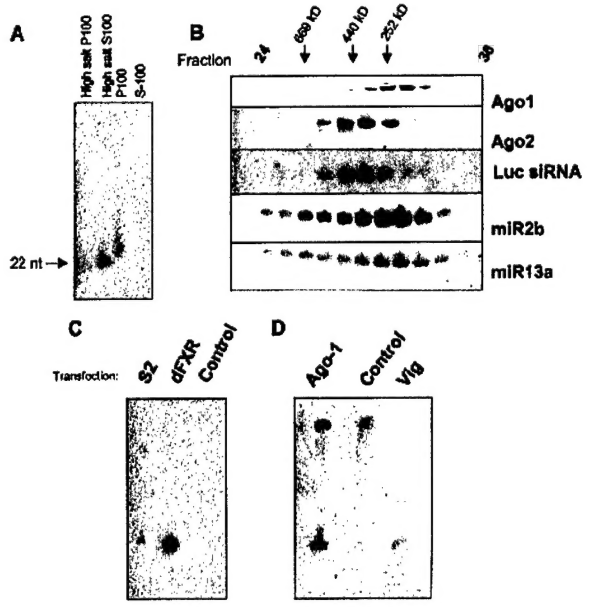

Figure 5. dFXR- [the *Drosophila* homolog of the Fragile X Mental Retardation Protein (FMRP)] and VIG-based RISC complexes contain microRNAs (miRNAs). (*A*) S2 cells were lysed hypotonically, and the nuclei were removed by centrifugation. The supernatant was pelleted at low salt, producing a S100 and P100. The P100 was extracted with high salt and centrifuged again to yield a high-salt P100 and a high-salt S100. RNA was extracted from equivalent portions of each and analyzed by Northern blotting with an oligonucleotide complementary to the *Drosophila* miRNA 13a. (*B*) S2 cells were soaked in luciferase double-stranded RNA, and extracts were prepared. Ribosomes were pelleted, salt extracted, and precipitated under low-salt conditions. The precipitates were dissolved again at high salt and size-fractionated on Superose 6. Column fractions were analyzed by Western blotting for Argonaute-1 (Ago-1) and Argonaute-2 (Ago-2) and by Northern blotting for miR2b, mir13a, and luciferase RNA. (*C*) S2 cells were transfected with T7-tagged FXR and his-tagged Vig expression constructs and size-fractionated. Fractions corresponding to the Ago-2 peak were immunoprecipitated using anti-T7 agarose beads and analyzed by Northern blotting for miR2b. Control immunoprecipitations were from similarly fractionated untransfected cells. (*D*) Drosophila S2 cells were transfected with the indicated tagged expression constructs or a GFP control. Proteins were collected by immunoprecipitation using anti-tag agarose beads. The beads were extracted with Trizol to prepare RNA and Northern blotted for miR2b.

The forgoing data suggest that miRNAs associate with RISCs that also contain dFXR and VIG. This is consistent with recent data suggesting that miRNAs can direct cleavage by RISC, given appropriate homologous substrates (Hutvagner and Zamore 2002). However, the observation of an ~250-kD peak containing the majority of miRNAs raised questions regarding the composition of these smaller complexes. Another Argonaute family member, Ago-1, showed co-fractionation of this protein with the major miRNA peak both on sizing columns (Fig. 5B) and subsequently on ion exchange columns (data not shown). To test the association of Ago-1 with miRNAs, we performed immunoprecipitation experiments with an epitope-tagged Ago-1 protein. Both mir2b and mir13a could be detected in association with Ago-1 (Fig. 5D; data not shown). Furthermore, Ago-1 immunoprecipitates also contained siRNAs (Fig. 2F). Reciprocal immunoprecipitations and Western blots indicate that dFXR and Vig do not detectably associate with Ago-1 (data not shown).

The forgoing data raises the possibility that siRNAs and miRNAs are distinguished to some extent and preferentially, although not exclusively, assembled into separate, functional effector RNPs. Recently, it has been shown that Ago-1 is required for efficient interference with endogenous genes in *Drosophila* embryos (Williams and Rubin 2002). Notably, Ago-1 complexes in S2 cells are similar in size to the active effector complexes, RISC*, that have previously been detected in embryo lysates (Nykanen et al. 2001). Our failure to detect substantial RISC activity in Ago-1 complexes and our failure to detect a substantial dependence on Ago-1 for RNAi in S2 cells could reflect developmental differences in the utilization of Ago family members or experimental differences in the assays used to detect RISCs, namely measuring loss of substrate (in our case) versus endonucleolytic cleavage (as in Nyanken et al. 2001).

The identification of the *Drosophila* homolog of the fragile X protein as a RISC subunit suggests that disruptions in RNAi-related pathways may contribute to human disease. The fragile X protein has previously been implicated in the regulation of gene expression at the level of protein synthesis (Brown et al. 2001; Darnell et al. 2001). In *Drosophila*, a bona fide dFXR regulatory target has been identified as Futsch, a Map1B homolog (Zhang et al. 2001). Indeed, dFXR and Futsch are epistatic with respect to the neuronal defects of dFXR-mutant flies, although they are not epistatic with respect to circadian defects (Dockendorff et al. 2002). Many miRNAs in *Drosophila* show complementarity to common sequence motifs that negatively regulate gene expression at the posttranscriptional level (Lai 2002). These K boxes, Brd boxes, and GY boxes are present in numerous mRNAs. Notably, a K box and a GY box are found in the 3′-UTR of Futsch. However, we cannot yet definitively link a sequenced miRNA to suppression of Futch expression. In humans, FMRP has been isolated as a component of large cytoplasmic mRNA complexes and,

through this association, has been implicated in the regulation of numerous mRNAs. None of the several proteins known to bind FMRP has been implicated in RNAi. However, there are many possible resolutions to this seeming contradiction. First, FMRP could function through several completely independent mechanisms. Second, FMRP could be an accessory factor for rather than an integral component of RISCs, using RISCs for identifying its cognate targets that may be regulated via mechanisms that involve its interaction with other proteins. This would be consistent with the partial effects of dFXR depletions on RNAi efficiency in S2 cells.

The precise mechanisms through which FMRP is delivered to its regulatory targets is unclear. Previously, selection of random RNA oligonucleotides implicated that FMRP binds G-quartet structures that lie adjacent to short RNA helices (Brown et al. 2001; Darnell et al. 2001). This interaction required only the RGG motif and did not involve the KH domain. Thus, it was proposed that FMRP recognized and regulated G-quartet-containing mRNAs. Here, we propose an alternative mechanism of regulation by fragile X protein. Our results are consistent with FMRP1 being targeted to its substrates as part of RNAi complexes, which are guided by miRNAs. Recognition of G-quartets might be an important determinant of further specificity or might be critical for translational regulation. We further propose that FMRP might be one of many distinct protein subunits that join RISCs, depending on the tissue, subcellular localization, and the developmental stage. In this way, RISCs could function as a flexible platform on which might be constructed a variety of regulatory machines that alter gene expression at the level of mRNA metabolism, at the level of protein synthesis, and at the level of genome structure.

Materials and methods

Cloning and expression

Ago-1, dFxr, and VIG coding sequences were amplified from full-length cDNAs by PCR, with N-terminal (Ago-1) or C-terminal (VIG, dFxr) epitope tags introduced. T7 (Novagen), Flag M2(Sigma), and hexahistidine tags were introduced, as indicated in the figure legends. PCR products were cloned into the pMT vector (Invitrogen), a $CuSO_4$-inducible insect expression vector. The I307N dFxr mutant was made using the Stratagene QuikChange kit. *Drosophila* S2 cells were cultured and transfected as described. (Hammond et al. 2000)

Immunoprecipitation

Transfected cells were lysed using 0.5% NP-40, 150 mM NaCl, 20 mM Hepes (pH 7.0), 2 mM $MgCl_2$, and 1 mM DTT. Proteins were immunoprecipitated using anti-T7 beads (Novagen) or anti-Flag M2 beads (Sigma). Immunoprecipitates were washed in the IP buffer containing 400 mM NaCl to extract ribosomes and were processed; Western blotting was as previously described (Hammond et al. 2001a). For Northern blotting, RNA from immunoprecipitates was extracted with Trizol (Invitrogen), separated on a 15% denaturing urea 1× TAE polyacrylamide gel, and electroblotted to Hybond N. Membranes were UV crosslinked and hybridized at 42°C in moderate stringency hybridization buffer (10 g/L BSA, 1 mM EDTA, 7% SDS, 15% formamide, and 0.4 M $NaPO_4$ at pH 7). Washes were at 42°C in 1× SSC, 0.1% SDS. For siRNA immunoprecipitations, S2 cells were transfected with uniformly ^{32}P-labeled dsRNA corresponding to the first 800 bp of the GL2 luciferase gene (Invitrogen) and the indicated expression construct. For RISC activity immunoprecipitations, transfected cells were immunoprecipitated as above, but the final wash was with buffer F (2 mM $MgCl_2$, 20 mM Hepes at pH 7, 110 mM KOAc, and 1 mM DTT). The beads were left in a small volume of buffer F, and ATP was added to 1 mM, RNAsin was added to 20 U/mL, and 1000 cpm of ^{32}P-labeled synthetic RNA transcript (as indicated) was added. Beads were removed at the indicated time points, and RNA was extracted with Trizol.

RISC purification

For protein identification, RISC activity was purified ~10,000-fold from *Drosophila* S2 cells, as described in Hammond et al. (2001a). Column fractions were TCA precipitated and run on a 4%–20% SDS-PAGE gradient gel. Protein bands were excised and analyzed by Matrix Assisted Laser Desorption Ionization and Liquid Chromatograph Quadropole Mass Spectrometry.

RNAi of RNAi

Drosophila S2 cells were transfected with 4 µg of a mixture of 500 nt dsRNAs covering the entire indicated cDNA. Three days after transfection, cells were further transfected with 4 µg of a 10:1 mixture of pGL3 control firefly luciferase expression vector (Promega) and pRLSV40 *Renilla* luciferase expression vector (Promega) and 25 ng dsRNA corresponding to firefly luciferase or GFP. Three days later, luciferase activity was measured using the Dual Luciferase kit (Promega). Ratios of firefly activity to *Renilla* activity were calculated. For each targeted gene, the expression in the cells undergoing RNAi against luciferase was normalized against control cells undergoing RNAi against GFP.

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